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Functional Uncoupling of the Janus Kinase 3-Stat5 Pathway in Malignant Growth of Human T Cell Leukemia Virus Type 1-Transformed Human T Cells

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Human T cell leukemia virus type 1 (HTLV-1) is a retrovirus that serves as the etiological agent for adult T cell leukemia, an aggressive and frequently fatal malignancy (1–3). In vitro studies have demonstrated that the virus dramatically alters T lymphocyte growth patterns (4), while infected patients generally display clonal expansion of CD4+ (5, 6) and CD8+ T lymphocytes (7). HTLV-1-mediated transformation or transition from a cytokine-dependent to an independent phase of growth has been attributed to Tax, a 40-kDa regulatory phosphoprotein encoded by the HTLV-1 virus (8–11). Tax has been shown to induce the transcription of several genes involved in promoting cell growth by enhancing degradation of IkB-α and IkB-β, thereby activating NF-κB family members (12, 13), which subsequently promote the expression of IL-2 and its receptor (14), c-fos and c-jun proto-oncogenes (15), and Stat5 (16) as well as associate with mitogen-activated protein/extracellular signal-related kinase kinase (17) and Ras (Ref. 9; for review, see Ref. 18).

Recent evidence suggests that HTLV-1 may also promote constitutive activation of a family of cytokine-activated signaling proteins known as Janus tyrosine kinases (Jaks) and Stat transcription factors. Under normal conditions, polypeptide hormones and cytokines induce transient tyrosine phosphorylation of these latent signaling molecules. Current models hold that oligomerization of the receptor complex by growth factors results in autophosphorylation of receptor tyrosines, with the receptor complex becoming activated and dimerizing, facilitating their dimerization and disengagement from the receptor complex upon which they migrate to the nucleus, bind select promoter elements, and ultimately regulate cell growth and differentiation (19). Serine kinases also regulate Stat protein activity. Specifically, serine residue S727 of Stat1, Stat3, and Stat5 becomes phosphorylated on serine residues following transcriptional activation (20–22). We recently reported that Stat5a and Stat5b become phosphorylated on serine residues following activation of lymphocytes by prolactin or IL-2 (23, 24). We subsequently mapped these sites to S726 in Stat5a and S731 in Stat5b (25). In T and B lymphocytes Stat5a/b are activated by the cytokine-restricted enzyme, Jak3. Jak3 is exclusively activated by the cytokines that use the common IL-2R chain (γc) and include IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 (19, 26–29). Mutations or
deletions that disrupt Jak3 activation and/or its association with the γc-chain are manifested as severe combined immunodeficiency syndrome in humans and mice (26). Moreover, activation of Stat5a and Stat5b has been determined to be unconditionally required for IL-2-mediated T cell proliferation based upon gene deletion experiments (30).

Whereas Jak and Stat proteins are normally unphosphorylated and inactive in quiescent lymphocytes, HTLV-1-transformed cells, including HuT-102 and MT-2, typically show constitutive tyrosine phosphorylation of Jak3 and Stat5 (31–33). Similarly, lymphocytes obtained from HTLV-1-infected patients display hypertyrosine-phosphorylated Jak3, Stat5, and Stat3, but not Jak2, Jak1, or Tyk-2 (34). Constitutively active Jak and Stat proteins are also commonly observed in a number of other malignancies, including Src-, v-Abl-, and EBV-transformed cell lines and patient lymphocytes (35–41). However, a causal relationship between malignant growth has not been established, nor is it clear whether disruption of this Jak/Stat signaling pathway will reverse the phenotypic condition.

We have recently reported that tyrphostin AG-490, a derivative of benzylidene mononitrile that resembles erbsbatin moiety, is a potent inhibitor of the Jak3/Stat5 signaling pathway in human and murine T cells (42–45). While this drug also has been shown to have effects on Jak2, it fails to inhibit Jak1, Tyk2, or a series of tyrosine kinases expressed in lymphocytes, including Lck, Lyn, Btk, Syk, Src, and Zap70 (43, 46). The studies described in this report were aimed at investigating whether AG-490 could preferentially inhibit constitutive and cytokine-induced activation of the Jak3/Stat5 signaling cascade within HTLV-1-transformed human T cells and subsequently disrupt malignant cell growth.

Materials and Methods

Cell culture and treatment

Freshly explanted human T lymphocytes were obtained from normal donors, purified by isocentrifugation, and activated for 72 h with 1 μg/ml PHA in RPMI 1640 medium containing 10% FCS (catalogue no. F2442, Sigma, St. Louis, MO), 2 mM l-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively). MT-2 and HuT-102 HTLV-1-infected cell lines were maintained in the above medium without PHA. T lymphocytes were made quiescent by washing and incubating for 24 h in RPMI 1640 medium containing 1% FCS before exposure to cytokines. Cells were treated with varying concentrations of AG-490 (catalogue no. 658401, Calbiochem, San Diego, CA) for 16 h as described in the figure legends. Cell lines were maintained in the above medium without PHA. T lymphocytes were made quiescent by washing and incubating for 24 h in RPMI 1640 medium containing 1% FCS before exposure to cytokines. Cells were treated with varying concentrations of AG-490 (catalogue no. 658401, Calbiochem, San Diego, CA) for 16 h as described in the figure legends. Cells were then incubated with 100 μM human IL-2 (Hoffmann-La Roche, Nutley, NJ), TNF-α, or IL-15 (PeproTech, Rock Hill, NJ) at 37°C as indicated in the corresponding figure legends. Cell pellets were frozen at −70°C.

Proliferation assays

Quiescent T, MT-2, and HuT-102 cells (5 × 10^4/well) were plated in flat-bottom 96-well microtiter plates in 200 μl of growth medium (described above) with 1% FCS. Cells were treated for 16 h with AG-490 or tyrphostin A25, B44, B46, B48, or B50 (Calbiochem, San Diego, CA) as described above, pulsed for the remaining 4 h of the assay with [3H]thymidine (0.5 μCi/200 μl), and harvested onto glass-fiber filters. ['^3H]Thymidine incorporation was analyzed by liquid scintillation counting (45).

Solubilization of membrane proteins and immunoprecipitation

Frozen cell pellets were thawed on ice and solubilized in lysis buffer (10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). Cell lysates were rotated end-over-end at 4°C for 60 min, and insoluble material was pelleted at 12,000 × g for 20 min. Depending on the experiment, supernatants were incubated with 5 μg/ml polyclonal rabbit antiserum raised against carboxy-terminal peptides corresponding to Stat5a (LDARLPPAGLFTSARSSL5) or Stat5b (MDQSWPHIAQ5S). Site-specific anti-Stat5 phosphoserine Abs were similarly produced to the corresponding phosphopeptide sequence QAAPG(PS)P, and all peptides were conjugated to keyhole limpet hemocyanin and used as immunogens in rabbits (25). Rabbit anti-Jak3 was prepared as previously described (28), and anti-phosphotyrosine or anti-phosphorytrosine Stat5a/b (Y694/699) mAbs were obtained from Upstate Biotechnology (Lake Placid, NY; catalogue no. 05-321 and 05-495, respectively). Abs were captured by incubation for 45 min with protein A-Sepharose beads (Pharmacia, Piscataway, NJ). Precipitated material was eluted by boiling in SDS-sample buffer for 4 min and was subjected to 7.5% SDS-PAGE under reducing conditions. All proteins were transferred to polyvinylidene difluoride membrane (catalogue no. IPVH00100, Immobilon, Millipore, Bedford, MA). All Western blots used the above described Abs diluted 1/1000 in blocking buffer for 1 h, while phosphospecific Stat5 Abs were blotted at 1/5000 for 20 h at room temperature.

EMSA

AG-490-treated and control (DMSO-treated) cells were pelleted by centrifugation (20,000 × g for 1 min at 4°C) and subsequently washed in 5 vol of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 100 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin; centrifuged; then lysed in the same buffer supplemented with 1% Nonidet P-40 and incubated for 20 min on ice. The nuclei-containing pellet was resuspended in equal volumes of low salt buffer (10 mM HEPES, 25% glycerol, 1.5 mM MgCl2, 20 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and protease inhibitors) and high salt buffer (low salt buffer containing 800 mM KCl). This fraction was then isolated by centrifugation at 4°C for 10 min, and supernatants were saved as nuclear protein extract and stored at −70°C. Gel mobility shift assays were performed to detect Stat5a/b DNA binding activity using a Stat5 DNA binding sequence corresponding to the promoter of the β-casein gene (5′-AGAGTTCGGAGAGCTTCCACG-3′) or an NF-κB binding element (5′-AGTTGAGGGACCTTCGAG-3′). Both probes were end labeled with [γ-32P]ATP. Labeled oligonucleotides were then incubated with 5 μg of nuclear-extracted proteins in 15 μl of binding cocktail (50 mM Tris-Cl (pH 7.4), 25 mM MgCl2, 5 mM DTT, and 50% glycerol) at 4°C for 2 h. For supershift assays, nuclear extracts were preincubated with 1 μg of either normal rabbit serum or antisera specific to Stat5a, Stat5b, or p50/p65 NF-κB (catalogue no. sc-1190X and sc-372X, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) as indicated in the figure legends at 4°C for 1 h, then incubated with [γ-32P]-labeled DNA oligonucleotide for 15 min at room temperature. The DNA-protein complexes were resolved on 5% polyacrylamide gels containing 0.25× TBE buffer, which were prerun in 0.25× TBE buffer for 1 h at 100 V. Samples were loaded, and gels were run at room temperature for approximately 2 h at 150 V, then dried by heating under vacuum and exposed to x-ray film (X-Omat, Eastman Kodak, Rochester, NY) at −70°C.

Results

AG-490 blocks constitutive tyrosine phosphorylation of Jak3 and Stat5a/b isolated from HTLV-1-transformed human T cells

Previously we had established that 75 μM of the Jak3 inhibitor AG-490 potentely inhibited IL-2-induced cell growth of the murine T cell clone, D10 (42). We next wanted to assess whether AG-490 also could block activation of Jak3/Stat5a/b in HTLV-1-transformed HuT-102 and MT-2 cells that display constitutively tyrosine-phosphorylated forms of both proteins. For this analysis actively growing HuT-102 and MT-2 cells were equally distributed and cultured with the drug for 16 h, then stimulated in the absence or the presence of IL-2 for 10 min. Jak3 and Stat5a/b were immunoprecipitated from cell lysates and subsequently blotted with anti-phosphoantibodies specific to anti-Jak3 (4G10) or anti-phosphotyrosine Stat5 (Y694/699), respectively (Fig. 1). Whereas each of the three signaling molecules displayed constitutive tyrosine phosphorylation in MT-2 (Fig. 1A) and HuT-102 (Fig. 1B) cells (lanes a, e, and i), AG-490 potently inhibited basal and noncytokine-inducible tyrosine phosphorylation of Jak3 (lanes c and d) and constitutive and IL-2-mediated tyrosine phosphorylation of Stat5a/b (lanes g, h, k, and l) in both cell lines. Densitometric analysis and normalization of constitutively tyrosine-phosphorylated Jak3 compared with total
protein were inhibited by 64 and 71% for MT-2 and HuT-102 cells, respectively, while Stat5α/b showed a corresponding loss of phosphorylation signal (data not shown).

HTLV-1 transformed T cells display constitutive serine-phosphorylated Stat5α (S726) and Stat5β (S731) that can be inhibited by AG-490 Stat1α and Stat3 are serine phosphorylated at residue S727, which is important for IFN-induced nuclear translocation, DNA binding, and maximal transcriptional activation (20–22). This site is located within a consensus PXSP phosphoacceptor site for mitogen-activated protein kinase (47, 48). Recent work by Zhang et al. (49) reported that phosphorylated S727 was also required for interaction with minichromosomal maintenance protein 5 and was necessary for maximum transcriptional activity and possibly cell cycle progression. Interestingly, IL-2 also inducibly regulates serine phosphorylation of Stat5α and Stat5β, and we have mapped this site to a serine residue of a PSP motif found in Stat5α and Stat5β (23, 25). To specifically analyze the phosphorylation status of this motif in Stat5α (S726) and Stat5β (S731) in HTLV-1-transformed cells, blots in Fig. 1 were next probed with site-specific phospho-serine Abs directed to phosphorylated peptide corresponding to aa residues 721–729 of human Stat5α or 726–734 of human Stat5β. Stat5α/b isolated from quiescent PHA-activated human T cells display serine phosphorylation of this site only upon IL-2 stimulation (data not shown). As depicted in Fig. 1, HuT-102 and MT-2 cells primarily displayed constitutively serine phosphorylation of Stat5α (lanes e and f), but not Stat5β (lanes i and j). AG-490 treatment significantly reduced Stat5α S726 phosphorylation without affecting total Stat5α protein levels (lanes g and h).

AG-490 disrupts basal and IL-15-inducible Jak3, Stat5α, and Stat5β tyrosine phosphorylation It has been previously reported that HuT-102 produces high levels of the IL-15 mRNA transcript, in contrast to nearly undetectable levels in resting or activated T cells (50). Bamford et al. (51) found that the R region, corresponding to the long terminal repeat of HTLV-1, is fused with the IL-15 transcript via the 5′ end of the untranslated region, driving its transcription and possibly promoting cell proliferation via an autocrine regulated loop. IL-15, like IL-2, uses the IL-2Rβ and γc-chains to activate Jak3 and Stat5; thus, we investigated whether AG-490 could also disrupt stimulation of Jak3/Stat5α/b by exogenously added IL-15 in these cells. As shown in Fig. 2 (upper panel), IL-15 increased the tyrosine phosphorylation of Stat5α (lanes e and f) and Stat5β (lanes i and j) over basal levels, but not that of Jak3 (lanes a and b). AG-490 treatment completely abolished basal and IL-15-mediated tyrosine phosphorylation of all three signaling proteins (lanes c, d, g, h, k, and l). Normalization of Jak3 and Stat5α/b phosphorysine levels compared with total protein indicated >70% loss of phosphorylation signal (data not shown).

FIGURE 1. AG-490 inhibits constitutive tyrosine phosphorylation of Jak3 and tyrosine-serine phosphorylation of Stat5α/b in HTLV-1-transformed T cells. MT-2 cells (A) or HuT-102 cells (B) were pretreated with DMSO (control) or 75 μM AG-490 for 16 h and then stimulated for 10 min with (+) or without (−) 100 nM IL-2 for 10 min at 37°C. Cells were lysed and then immunoprecipitated with anti-Jak3 (lanes a–d), blotted with antiphosphotyrosine, stripped, and reprobed with polyclonal Jak3 Abs. Alternatively, lysates were immunoprecipitated with Stat5α (lanes e–h) or Stat5β (lanes i–l), blotted with monoclonal antiphosphotyrosine Stat5, polyclonal phosphoserine Stat5 (S726-Stat5α or S731 Stat5β), and reblotted with monoclonal pan Stat5. Densitometry and normalization of Jak3 samples isolated from DMSO-treated compared with AG-490-treated cells showed decreased constitutive (without IL-2) tyrosine phosphorylation for MT-2 of 64% (A, lane a, of 0.671 relative absorbance units (rau), lane b of 0.803 rau, lane c of 0.239 rau, and lane d of 0.460 rau) and for HuT-102 of 71% (B, lane a of 0.825 rau, lane b of 0.608 rau, lane c of 0.240 rau, and lane d of 0.265 rau).
AG-490 inhibits constitutive and IL-2-induced activation of Stat5a/b DNA binding activity in HuT-102 and MT-2 nuclear cell extracts, but not NF-κB

Jak-mediated Stat tyrosine phosphorylation is required for dimerization, disengagement from the receptor, nuclear translocation, and subsequent gene transcription (19). To test the idea that AG-490 inhibits the generation of transcriptionally competent Stat5a/b, gel electrophoretic mobility shift assays were used to assess their ability to bind to an oligonucleotide probe corresponding to the Stat5 binding element of the β-casein gene promoter. Nuclear extracts isolated from tyrphostin-treated HuT-102, MT-2, or PHA-activated human T cells were harvested from samples identical to those depicted in Fig. 1 and incubated with 32P-labeled β-casein probe in the absence or the presence of specific Stat5a/b antisera (Fig. 3). Extracts from IL-2-stimulated cells treated overnight with DMSO displayed a weakly inducible, single DNA binding complex that could be supershifted with anti-Stat5a (lane c), partially with anti-Stat5b (lane d), or completely with both Stat5a and Stat5b Abs (lane e), but not with normal rabbit sera alone (lane f). To determine whether all transcription factors were equally inhibited by AG-490 in HTLV-1-transformed cells, the non-Jak3-activating pathway, TNF-α, was examined.

For this study (Fig. 4), HuT-102 (top panel), MT-2 (middle panel), or PHA-activated (bottom panel) primary T cells were treated with vehicle (lanes a–e) or AG-490 (lanes f–j) and stimulated in the absence (lanes a and f) or the presence (b–e and g–j) of 100 nM TNF-α for 10 min at 37°C. Next, 5 μg of nuclear cell extract from each sample set was incubated with the NF-κB DNA binding element and supershifted with the appropriate Ab to confirm the identity of the p50/p65 complex. AG-490 was not effective at inhibiting constitutive or cytokine-inducible NF-κB DNA binding activity (Fig. 4, lanes a–j).

AG-490 blocks IL-2-mediated growth of mitogen-activated human T cells, but not HTLV-1-transformed cells

Since the Jak3/Stat5 pathway is known to promote T cell growth, we next tested whether AG-490 could effectively block leukemic cell growth of HTLV-1-transformed cells (26, 30). Previously studies indicated that T cells treated with AG-490 were >90% viable, and inhibition of IL-2-inducible cell proliferation was recoverable (43). For these experiments, actively growing PHA-activated T cells, HuT-102, or MT-2 cells were cocultured with increasing concentrations of drug for a period of 16 h and assayed.

FIGURE 2. AG-490 inhibits constitutive and IL-15-inducible tyrosine-phosphorylated forms of Stat5a, Stat5b, and Jak3 in HuT-102 cells. Upper panel, HuT-102 cells were pretreated with DMSO (control) or 75 μM AG-490 for 16 h and stimulated for 10 min with (+) or without (−) 100 nM IL-15. Cells were lysed and then immunoprecipitated with anti-Jak3 (lanes a–d), Stat5a (lanes e–h), or Stat5b (lanes i–l) and blotted with antiphosphotyrosine. Lower panel, Reblot of the Western blot presented above that had been stripped and reprobed with a mixture of Abs directed to Jak3, Stat5a, or Stat5b to examine expression levels. Molecular weight markers are shown on the left, and arrows denote Jak3 or Stat5a/b.

FIGURE 3. Pretreatment of human T cells or HTLV-1-transformed HuT-102 or MT-2 cells with AG-490 inhibits constitutive and IL-2-induced Stat5a/b DNA binding, as demonstrated by EMSA analysis. Lower panel, PHA-activated T cells were treated as described in Fig. 2 with either DMSO (control; lanes a–f) or 75 μM AG-490 (lanes j–l) and incubated with medium (−) or 100 nM IL-2 (+) for 10 min at 37°C. MT-2 (middle panel) or HuT-102 (upper panel) cells were treated similarly, and nuclear extracts corresponding to 5 μg of protein were incubated in the absence of Ab (lanes a, b, g, and h) or with α-Stat5a (lanes c and i), α-Stat5b (lanes d and j), or normal rabbit serum (lanes e and k) in combination with a 32P-labeled oligonucleotide probe corresponding to the PRL response element of the β-casein gene promoter. Migrational location of nonsupershifted Stat5a/b DNA complexes before Ab addition are indicated by arrows on the right.
AJ. 490 fails to inhibit constitutive or TNF-α-inducible NF-κB DNA binding activity in HuT-102 and MT-2 cells. HTLV-1-transformed HuT-102 cells (top panel), MT-2 (middle panel), or primary human T cells (bottom panel) were pretreated with DMSO (lanes a–e) or 75 μM AG-490 (lanes f–j) in the presence or the absence of medium (–) or 100 nM TNF-α (+) for 10 min at 37°C and then subjected to DNA binding analysis using a radiolabeled probe (see Materials and Methods). For this assay 5 μg of nuclear cell extracts were incubated in the absence (lanes a, b, f, and g) or the presence of Ab to p50 (α-p50; lanes c and h), anti-p65 (α-p65; lanes d and i), or normal rabbit serum (lanes e and j) in combination with a 32P-labeled oligonucleotide probe corresponding to the NF-κB DNA-binding element (lanes a–j). Ligation of the nonsupershifted (without Ab) p50/p60 NF-κB (NF-κB) complex is indicated by an arrow on the right.

Discussion

In the present study we provide evidence that AG-490 blocks constitutive and IL-2/IL-15-induced tyrosine phosphorylation of Jak3 and Stat5a/b isolated from HTLV-1-transformed MT-2 and HuT-102 cell lines (Fig. 1). We also provide the first account that HTLV-1-transformed cells display constitutive serine-phosphorylated Stat5a (S726), but not Stat5b, and that this kinase was efficiently blocked by AG-490, most likely via inhibition of its far upstream activator, Jak3 (Fig. 1). EMSA analysis further verified that DNA binding of partially purified Stat5a/b harvested from actively growing or cytokine-stimulated MT-2 or HuT-102 cells was significantly inhibited following AG-490 treatment (Fig. 3). However, this drug had no overt effect on constitutive NF-κB DNA binding activity isolated from similarly treated cells (Fig. 4). Inhibition of the Jak3/Stat5a/b signaling pathway by AG-490 failed to disrupt leukemic growth of HTLV-1-transformed cells. Similarly, several other potent tyrosine kinase inhibitors were competent to block basal and IL-2-stimulated cell growth of mitogen-activated lymphocytes, but had no effect on HTLV-1-transformed cells (Figs. 5 and 6).

Jak and Stat proteins have received much attention as putative targets for controlling leukemogenesis. Indeed, constitutively activated Jak and Stat proteins have been identified and studied in several distinct oncogenic models, including leukemia-like phenotype in Drosophila (52), B cell Ag receptor-Abl-expressing cell lines and patient peripheral lymphocytes (40), acute myeloid leukemia blasts (53), B lymphocytes obtained from patients afflicted with chronic lymphocytic leukemia (54), EBV-infected (41), and src-transformed cells (35–39). The explanation of how the HTLV-1 virus transforms T cells to a state of growth factor independence is not readily apparent. However, it is known that...
infected T cell cultures become IL-2 independent over time, which parallels acquisition of constitutively active Jak3, but not Jak2, Jak1, or Tyk2 (34). Moreover, lymphocytes obtained from HTLV-1-infected patients display constitutively active Stat proteins, which also correlates with enhanced Jak3 activity (30). Since Jak3 is only recruited and activated by T cell growth factors that use \( g_c \), autocrine-regulated growth by one or more of these cytokines has been proposed (34). An autocrine-regulated loop for IL-2 or IL-15 was mostly dispelled, since coculturing cells with respective Abs failed to disrupt malignant cell growth or tyrosine phosphorylation of Jak and Stat proteins; however, other Jak3-activating cytokines (e.g., IL-4 or IL-7) could not be excluded (34). Here we provide evidence that AG-490 potently inhibits constitutive Jak3 activity. Previously we reported that this tyrphostin selectively inhibits IL-2-induced autophosphorylation of Jak3 following in vivo or direct in vitro treatment (43). Moreover, stimulation of Jak3 by the other \( g_c \) cytokines (IL-4, IL-7, IL-9, and IL-15) was profoundly blocked at concentrations of AG-490 similar to those used here (43). Since proliferation assays failed to detect any disruption in cell growth of MT-2 or HuT-102 following treatment with tyrophostin-derived tyrosine kinase inhibitors, PHA-activated human T cells, HuT-102 cells, and MT-2 cells were treated with 100 \( \mu \)M AG-490, A25, B44, B46, B48, or B50 for 16 h at 37°C (ordinate). All cells were then pulsed with \( [\text{3} \text{H}] \)thymidine (0.5 \( \mu \)Ci/200 \( \mu \)l) for 4 h, and incorporation of radiolabel is plotted on the abscissa, expressed as total counts per minute. Error bars represent the SEM (n = 6).

Can HTLV-1 promote oncogenesis by non-Jak tyrosine kinases? A recent report found that human T cells transformed by herpesvirus saimiri display constitutively activated Zap70 and p56\( \text{lk} \), but not Jak or Stat proteins (55, 56). Upon further investigation, we failed to detect tyrosine phosphorylation of either protein in MT-2 or HuT-102 cells (data not shown). However, several of the more broadly based tyrphostins (A25, B44, B46, B48, and B50), which also inhibited Jak3 (R. A. Kirken, unpublished observation) might be expected to have an effect on the hyperactivation of additional tyrosine kinases. Nonetheless, all five agents blocked both basal and IL-2-induced proliferation of PHA-activated T cells, while only B46 showed moderate inhibition (~40%) at 100-\( \mu \)M concentrations of drug in HuT-102 cells; however, this response was

![FIGURE 5](image-url) AG-490 inhibits IL-2-induced proliferation of T cells in a dose-dependent manner, but not HTLV-1-transformed HuT-102 or MT-2 cell lines. Top panel, Cell proliferation of PHA-activated, quiescent T cells (5 \( \times \) 10^4/well) was examined following treatment with increasing concentrations of AG-490 (ordinate) for 16 h at 37°C in the presence of 1 nM IL-2, except for the sample represented by the first bar. Similarly, HuT-102 (middle panel) and MT-2 cells (bottom panel) were treated with DMSO or AG-490 as indicated. All cells were then pulsed with \( [\text{3} \text{H}] \)thymidine (0.5 \( \mu \)Ci/200 \( \mu \)l) for 4 h, and incorporation of radiolabel is plotted on the abscissa, expressed as total counts per minute. Error bars represent the SEM (n = 6).

![FIGURE 6](image-url) Various tyrosine kinase inhibitors ablate cell growth of mitogen-activated T cells but not HuT-102 or MT-2 cells. Top panel, Proliferation of PHA-activated, quiescent T cells (5 \( \times \) 10^4/well) was examined following treatment with tyrophostin-derived tyrosine kinase inhibitors. PHA-activated human T cells, HuT-102 cells, and MT-2 cells were treated with 100 \( \mu \)M AG-490, A25, B44, B46, B48, or B50 for 16 h at 37°C (ordinate). All cells were then pulsed with \( [\text{3} \text{H}] \)thymidine (0.5 \( \mu \)Ci/200 \( \mu \)l) for 4 h, and incorporation of radiolabeled probe is plotted on the abscissa, expressed as total counts per minute. Error bars represent the SEM (n = 6).
not observed for MT-2 cells (Fig. 6). From this evidence we conclude that tyrosine kinases in general may not play a predominant role in the leukemogenesis of these cells.

The 40-kDa phosphotyrosine Protein Tax does not bind directly to DNA; however, it is responsible for driving the trans-activation and transcription of several proliferative genes in addition to HTLV-1, presumably by interacting with a variety of protein factors, including CREB/ATF binding proteins, p65SRF, and cyclin-dependent kinase inhibitor p16INK4A, allowing for cyclin-dependent kinase-4 activation and activation of NF-κB (18). NF-κB is normally sequestered in the cytoplasm of resting T lymphocytes; however, Tax stimulates the phosphorylation and degradation of IκB-α and IκB-β via two conserved serine residues that allow transcriptionally active NF-κB to translocate to the nucleus and promote expression of IL-2 and IL-2R α-chain (14), c-fos and c-jun proto-oncogenes (15), and Stat5 (16). It is of interest to note that these signaling pathways are predominantly dependent on serine-threonine, and not tyrosine kinase, activity. As stated above, several tyrosine kinase inhibitors failed to block the growth of HTLV-1-transformed cells (Fig. 6). Despite potent inhibition of Stat5αβ DNA binding by AG-490, there was no loss of constitutive NF-κB DNA binding activity (Figs. 3 and 4). Taken together, it seems likely that therapeutic approaches aimed at ablating Tax/serine-threonine kinase signaling molecules may hold greater potential for treating this disease. Whether similar observations and conclusions will be encountered for other leukemic malignancies displaying hyperphosphorylated Jak and Stat signaling proteins remains to be determined.

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