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Combinatorial approach to identification of tyrphostin inhibitors of cytokine signaling

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Abstract—Aberrant or deregulated activity of certain cellular kinases has been shown to cause certain malignancies and other disorders. The tyrphostin molecule AG490 inhibits the action of the janus kinases JAK2 and JAK3. JAK2 is an indispensable molecule for transducing the signals conveyed by a large number of cytokines including IL-3 while JAK3 is essential for signaling by a smaller number of cytokines including IL-7. A synthetic combinatorial chemical library containing 599 compounds was created and screened for the ability to inhibit proliferation of IL3- and IL7-dependent cell lines to focus on molecules that interrupt those signaling pathways. This screen identified a *meta*-trifluoromethyl derivative of AG490, **5H4**, that is approximately twice as potent as AG490 in cell-based assays. **5H4** blocked the factor-dependent proliferation of both of these cell lines, actively promoted cell death, and diminished the JAK kinase activity. Administration of **5H4** to lymphoma-prone IL-7 transgenic mice reduced their spontaneous lymphadenopathy. The improved characteristics of this novel compound bring this class of molecules closer to therapeutic utility.

1. Introduction

The four members of the janus (JAK) kinase family, JAK1, JAK2, JAK3, and TYK2 are non-receptor protein tyrosine kinases that play essential roles in the cellular responses to distinct cytokines. JAK kinases comprise FERM, SH2, pseudokinase, and kinase domains. The FERM domain mediates receptor interactions and both the FERM and pseudokinase domains regulate the catalytic activity of the kinase domain.[1–4] SH2 domains bind to certain protein domains containing phosphorylated tyrosine residues.[5] JAK1, JAK2, and TYK2 appear to be expressed ubiquitously and are each activated by several different cytokines while JAK3 expression is normally restricted to lymphoid cells.

The cellular responses to cytokines are important components of normal developmental and immunological processes, however, they can also contribute to pathological and malignant cellular behavior. A body of evidence has accumulated implicating abnormal constitutive activation of JAK kinases in various cancers. This activation may be as a result of persistent ligand expression, viral infection, chromosomal rearrangement, or other unknown mechanisms. For example, IL-7 and IL-15 convey potent mitogenic signals and enhance cell viability by activating JAK3, which induces expression of an array of anti-apoptotic genes including Bcl-2.[6,7] Forced autocrine expression of IL-7 or IL-15 in transgenic mice causes inflammatory disorders and hematopoietic malignancies[8–10] and persistent IL-7 signaling has been implicated in a number of human malignancies including cutaneous T cell lymphomas (CTCL),[6] acute T cell leukemias,[7,11] B cell lymphocytic leukemia,[12–14] Burkitt's lymphoma,[15] Hodgkin's disease,[16] and melanoma.[17] Infection of certain cells with HTLV-I activates JAK kinases in T lymphocytes and leads to their transformation.[18,19] Translocations between the TEL gene and JAK2 are found in T cell[20] and myeloid[21] leukemias. These translocations fuse the helix–loop–helix domains of the TEL

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gene with the kinase domain of JAK2. The TEL portion of the resulting fusion protein causes it to oligomerize spontaneously leading to constitutive activation of the kinase in the JAK2 portion.

Several types of small molecules that inhibit the activity of JAK kinases and block cytokine signal transduction have been identified and evaluated as potential therapeutic agents. These include the tyrphostin family of tyrosine analogs,[22,23] a natural product, cytovaricin B,[24] octylamino-undecyl-dimethylxanthine compounds,[25] derivatives of dimethoxyquinazoline,[26] some naphthyl ketones,[27] prodigiosin derivatives,[28] and a pyridone-containing-tetracycle[29] as well as the recently described CP-690,550.[30] The best studied class of these inhibitors is the tyrphostin family.[31,32] These molecules are characterized by an alkene substituted with a nitrile and a hydroxylated phenyl group in a cis configuration (see Fig. 1A). Tyrphostin AG490 (also known as B42) inhibits both JAK2 and JAK3 kinases and prevents phosphorylation of phosphatidyl inositol-3 kinase. AG490 blocks the signal transduction of a broad range of cytokines that utilize JAK2 or JAK3 including IL-2, IL-3, IL-7, IL-9, IL-12, IL-15, and GM-CSF.[31,33,34] Although as a potential Michael reaction acceptor AG490 might be expected to react with cellular components, similar molecules have been shown to be purely competitive inhibitors for the tyrosine-containing substrate and non-competitive or mixed-competitive inhibitors with respect to ATP.[35] AG490 blocks the growth of a number of different malignant cells including the K562 chronic myelogenous leukemia cell line,[36] and malignant cells from acute lymphoblastic leukemia,[31] mycosis fungoides,[37] myeloma,[38] Sezary syndrome,[39] large granular lymphocytic leukemia,[40] Hodgkin's disease,[41] and Philadelphia-positive B-lineage leukemia.[42] JAK3 and the v-abl protein interact with one another[43] and AG490 has been shown to have an additive effect on the antiproliferative activity of STI571 on BCR-ABL driven proliferation.[44]

In this study we have utilized cell-based assays to screen for inhibitors for JAK2 and JAK3 among a combinatorial chemical library comprising 599 variants of AG490.[45] Two cytokine-dependent murine cell lines, Baf/3 (an IL3-dependent pro-B cell line utilizing JAK2 kinase) and 2E8 (an IL7-dependent pre-B cell line utilizing JAK3 kinase), were used in the screening. We identified several small molecules that inhibit the proliferation of 2E8 and Baf/3 cells more potently than AG490. The two most potent of these, containing trifluoromethyl groups, were obtained in larger quantities

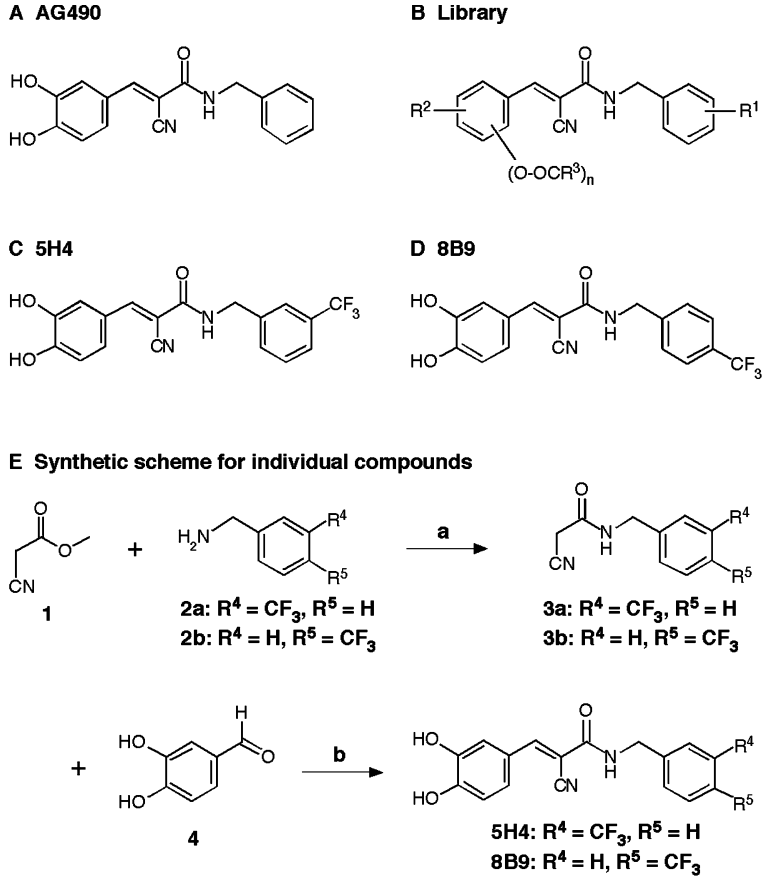

Figure 1. Structures of compounds. (A) Tyrphostin AG490, also known as B42. (B) Variation of the combinatorial chemical library. (C) Compound **5H4**. (D) Compound **8B9**. (E) Synthetic scheme for individual compounds. Reagents and conditions: (a) 100 °C, 6 h; 75% (**3a**), 73% (**3b**); (b) piperidine, EtOH, reflux, 1 h; 82% (**5H4**), 75% (**8B9**).

and characterized. These new inhibitors of JAK2 and JAK3 may be useful as therapeutic agents to treat kinase-mediated malignancies or other disorders.

2. Results

2.1. Sensitivity of cytokine-dependent cells to variants of tyrphostin AG490

The IL7-dependent 2E8 cells that require activated JAK3 for growth and the IL3-dependent Baf/3 cells that require activated JAK2 were each used to screen the library of AG490 variants. Cells were cultured in the presence of the appropriate cytokine and each of the 599 compounds from the chemical library as well as AG490 and proliferation was measured as ^{3}H-thymidine incorporation. Control cultures containing DMSO only or no cytokine were also analyzed in parallel. Screens of the compounds at 10 μM, in which AG490 inhibited proliferation by less than 50%, were most informative. The two most potent candidate compounds, **5H4** and **8B9**, were substantially better inhibitors of both Baf/3 and 2E8 cells than AG490. Samples of these two molecules from the combinatorial library were reproducibly 4–8 times more potent than samples of AG490 synthesized in parallel. Remarkably, these two compounds **5H4** and **8B9** are similar *meta*- and *para*-trifluoro-methyl substitutions of AG490, respectively (see Fig. 1C and D).

To facilitate further characterization of molecules **5H4** and **8B9**, gram quantities of each were prepared. Figure 1E summarizes the syntheses of **5H4** (NSC D722757), and **8B9** (NSC D722756). The procedures used were based on those for similar compounds described in the literature.[46] By heating methyl cyanoacetate and the appropriately substituted benzylamine **2a** or **2b**, the desired α-cyanoamide **3a** or **3b** is obtained in good yield. The α-cyanoamide is then condensed with 3,4-dihydroxybenzaldehyde in the presence of piperidine to give the desired tyrphostins **5H4** and **8B9** in good yield. Final compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, and MS.

These preparations were then evaluated for their abilities to inhibit JAK-dependent proliferation of the 2E8 and Baf/3 cell lines as well as the Jurkat human acute T cell leukemia cell line and the HL-60 human acute promyelocytic leukemia cell line. The results of these assays are depicted in Figure 2 and the concentrations at which each of these preparations inhibit 50% of

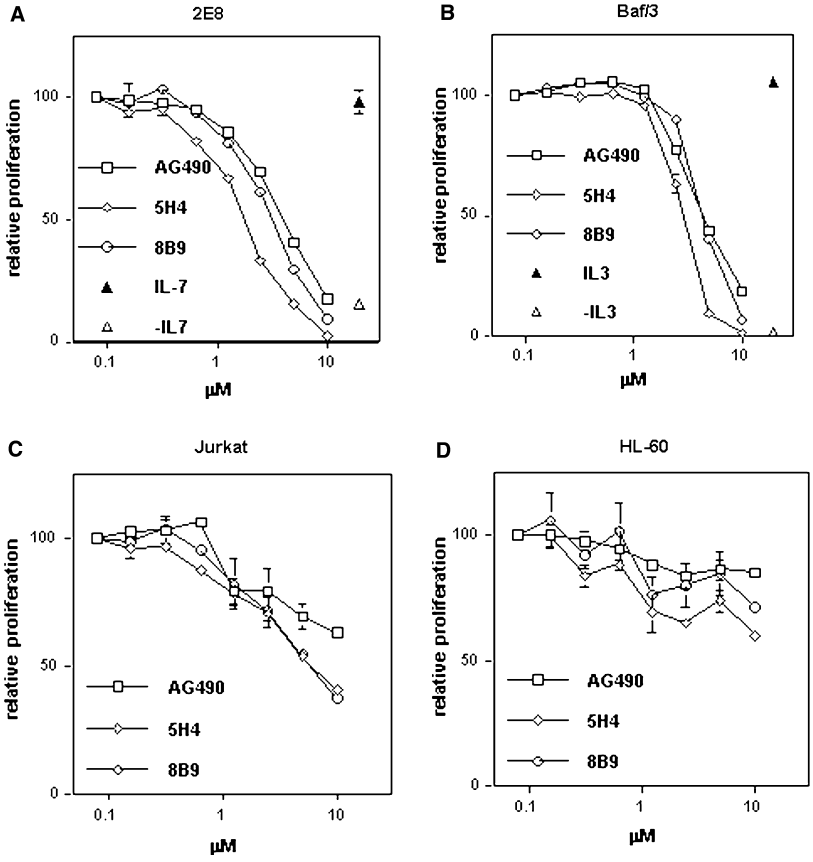

Figure 2. Inhibition of cell proliferation by tyrphostins. Cell lines were cultured in the presence of increasing concentrations of the indicated compounds and proliferation was measured as ^{3}H-thymidine incorporation. Concentrations of compounds are indicated on the *x*-axis. ^{3}H-thymidine incorporation values are plotted on the *y*-axis as a percentage of the values obtained in the absence of added compound or the lowest dilution (0.08 μM) and in the presence of an optimal concentration of the requisite cytokine for cytokine-dependent cells (IL-7 for 2E8 and IL-3 for Baf/3). Each data point represents the mean of quadruplicate cultures. Error bars indicate the standard error. Isolated triangles on the right side of panels A and B indicate values obtained in the absence (open) or presence (filled) of IL-7 or IL-3, respectively.

Table 1. IC_{50} values (μM) for tyrphostins on different cell lines

Cell line	AG490	**5H4**	**8B9**
2E8	3.9 ± 0.06	1.7 ± 0.08	3.1 ± 0.1
Baf/3	4.6 ± 0.3	2.8 ± 0.02	4.5 ± 0.1
Jurkat	(37%)[a]	6.1 ± 0.4	5.9 ± 0.5
HL-60	(15%)[a]	(40%)[a]	(29%)[a]

[a] Inhibition at highest concentration tested (10 μM).

^{3}H-thymidine incorporation (IC_{50}) of each of the cell lines are listed in Table 1.

Jurkat cells, which are reported not to express JAK3,[33] were only moderately sensitive to all three compounds. Interestingly, the Jurkat cells were significantly more sensitive to **5H4** as well as **8B9** than they were to AG490 (Fig. 2C). This is in contrast to a previous observation that Jurkat cells were relatively insensitive to AG490.[33] HL-60 cells exhibited very little sensitivity to any of the compounds (Fig. 2D). Compounds **5H4** and **8B9** have also been screened for inhibition of growth of the NCI-60 panel of cell lines by the Developmental Therapeutics Program of NCI/NIH. The results of these assays can be obtained at http://dtp.nci.nih.gov/ under the names NSC D722757 (**5H4**) and NSC D722756 (**8B9**).

2.2. Induction of programmed cell death by novel tyrphostins

Since IL-7 supports the growth of CTCL by upregulating the anti-apoptotic gene Bcl-2[6] and AG490 can block cell growth by inducing apoptosis,[31,47] we next examined whether the candidates from the screening use the same mechanism of inducing cell death to block cell proliferation. 2E8 and Baf/3 cells were treated with AG490, **5H4**, or **8B9** at the concentration of 10 or 20 μM for 48 h. Since active degradation of chromatin is a hallmark of apoptosis, we measured the cellular DNA content as an index of programmed cell death. Cells were stained with propidium iodide and analyzed by flow cytometry. The percentages of cells with less DNA than diploid cells (sub-G1), representing dead cells, are shown in Figure 3. Withdrawal of IL-7 from 2E8 cells induced 50% cell death, whereas treatment with 20 μM of AG490, **8B9**, or **5H4** induced 60%, 71%, and 83% cell death, respectively (Fig. 3A). Similar data was obtained from Baf/3 cells. Withdrawal of IL-3 from Baf/3 cells induced 87% cell death, whereas treatment with 20 μM of AG490, **8B9**, **5H4** induced 49%, 75%, 74% of cell death, respectively (Fig. 3B). Consistent with the cell proliferation results (Fig. 2), **5H4** inhibits more of the cell growth by inducing more cell death. Control cultures contained 0.1% (14 mM) DMSO, which is the same concentration as those containing compounds at 20 μM. This is substantially lower than the concentrations of DMSO that inhibit the proliferation of 2E8 cells (IC_{50} = 150 mM) or Baf/3 cells (IC_{50}=260 mM) (data not shown).

2.3. Inhibition of JAK3 kinases by novel tyrphostins

Because JAK kinases are the critical mediators for IL-7 and IL-3 signaling, we next examined the effects of the compounds on kinase activities. 2E8 and Baf/3 cells were collected after 48-h drug treatment and lysates were prepared. JAK3 proteins were immunoprecipitated from these lysates and the extent of their phosphorylation was determined by immunoblot with anti-phosphotyrosine antibodies. As shown in Figure

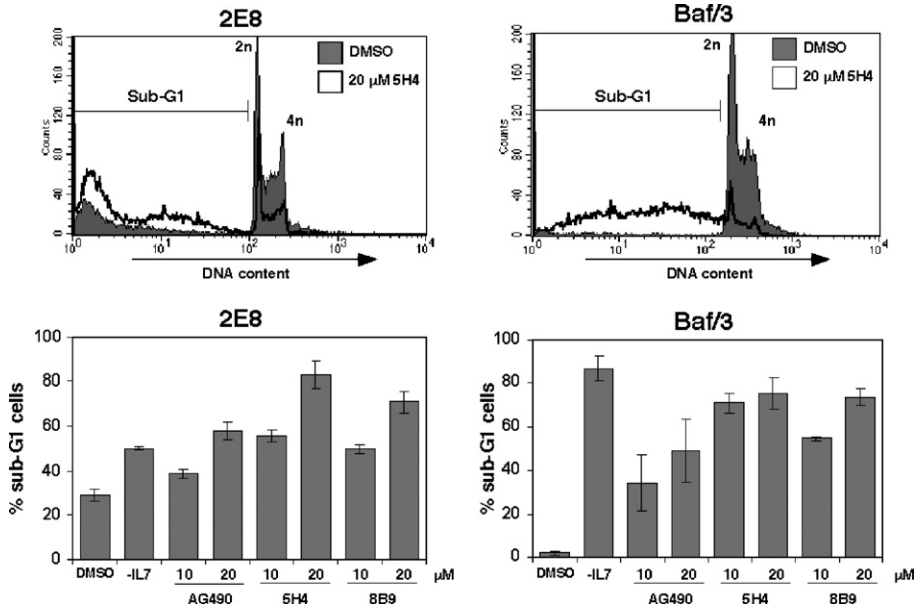

Figure 3. Induction of cell death by tyrphostins. 2E8 and Baf/3 cells were grown in the presence or absence of recombinant murine IL-7 or IL-3, respectively, and 0.1% DMSO with or without the indicated concentrations of tyrphostins for 48 h. Cells were then stained with propidium iodide and fluorescence was measured by flow cytometry to quantify DNA content. Representative histograms are shown in the upper panels. Bar graphs of the percentages of cells with fluorescent intensities lower than cells in phase G1 (sub-G1) representing dead cells in each condition are plotted in the lower panels.

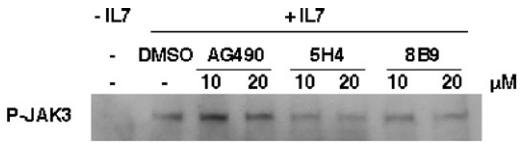

Figure 4. Inhibition of JAK kinase activity by tyrphostins. IL-7-dependent 2E8 cells were cultured without IL-7 or with IL-7 in the presence of the indicated compounds for 48 h. Whole cell extracts containing 500 μg of protein were immunoprecipated with anti-JAK3 antibody and the captured material was immunoblotted with anti-phosphotyrosine antibody.

4, phosphorylation of JAK3 was inhibited to a greater extent in lysates prepared from **5H4**- and **8B9**-treated cell than in lysates from AG490-treated cells.

2.4. Regression of lymphadenopathy in IL-7 transgenic mice by treatment with 5H4

Transgenic mice expressing IL-7 under the control of the immunoglobulin heavy chain enhancer and promoter (IL7TG) develop a lymphoproliferative disorder that progresses to B and T cell lymphomas.[8] Since JAK3 is an essential mediator for IL-7 signaling, we examined the effect of administration of compound **5H4** to IL7TG mice. Magnetic resonance imaging (MRI) was used to precisely measure the dimensions of lymph nodes of living IL7TG mice before and after treatment. As shown in Figures 5A and 6, IL7TG mice have greatly enlarged axillary lymph nodes relative to normal wild-type FVB mice and this can be clearly visualized by MRI.

Two independent experiments were performed in which the effects of **5H4** on IL7TG mice were evaluated. The first experiment involved eight mice and the second experiment involved four. In each experiment, 5–6 month old heterozygous IL7TG mice were treated with intraperitoneal injections of 0.1 mL of DMSO containing 1 mg of **5H4** or DMSO alone for 14 days. In the first experiment MRI images were collected of all mice before treatment. One IL7TG mouse died after two doses of 1 mg **5H4** injection on the first day of the first experiment. Subsequently, all treatments were reduced to one dose of 1 mg **5H4** per day. During the course of the first experiment another **5H4**-treated mouse and one DMSO-treated mouse died. Each of the surviving mice in the first experiment, two **5H4**-treated mice and three DMSO-treated mice, were analyzed by MRI and histology. In the second experiment, one DMSO-treated

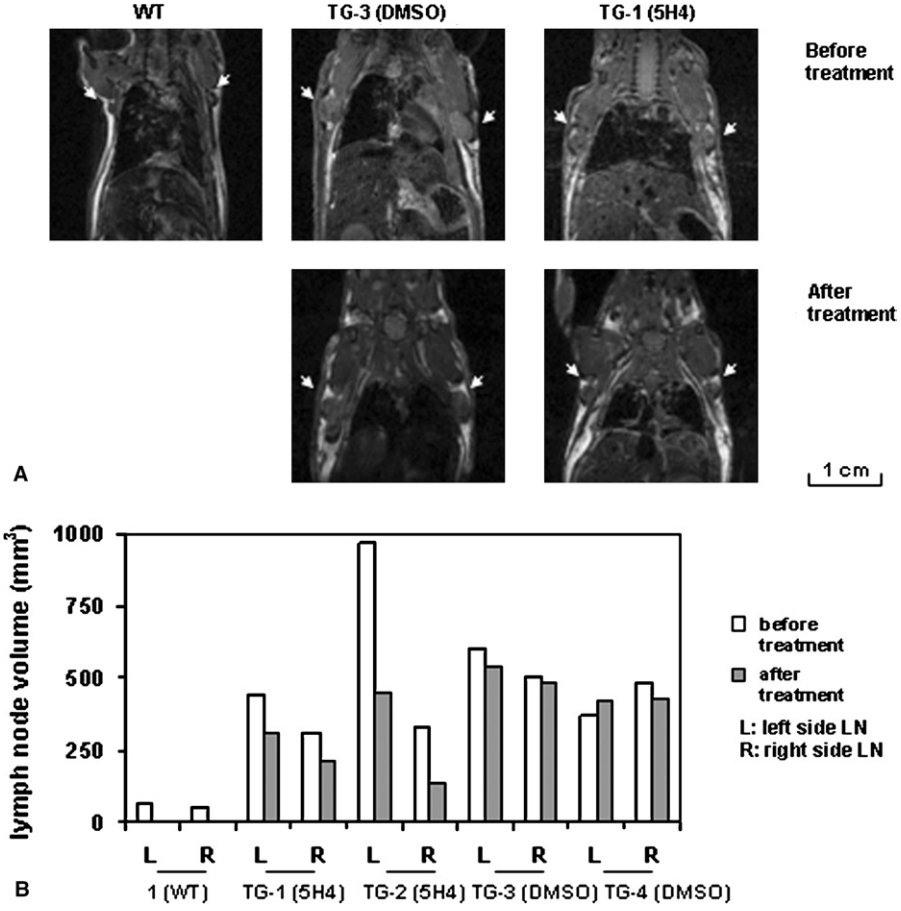

Figure 5. Treatment of IL7TG mice with tyrphostin 5H4. Groups of 5–6 month old heterozygous IL7TG mice were administered daily intraperitoneal injections of 1.0 mg **5H4** in DMSO, or DMSO alone for 14 days. MRI imaging was performed before and after the treatment. (A) Representative images of IL7TG mice (TG) treated with **5H4** in DMSO or DMSO alone as well as a matched wild-type mouse (WT) as indicated, are presented. The axillary lymph nodes are indicated by arrows. (B) Axillary lymph node volumes before and after treatment were measured and calculated as described in Experimental and plotted as white (before treatment) or shaded (after treatment) columns.

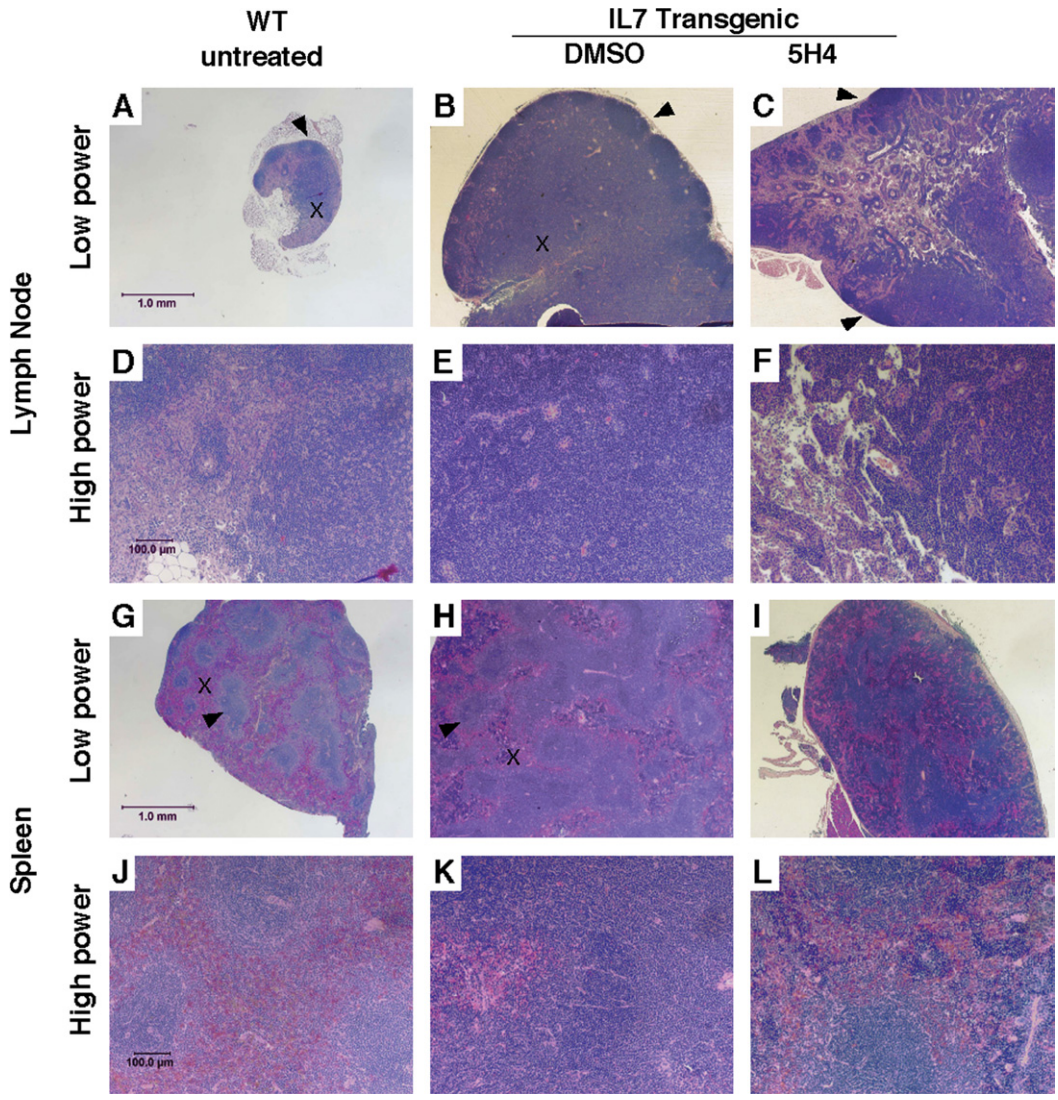

Figure 6. Histology of lymph nodes and spleen. Hematoxylin and eosin stained paraffin sections of lymph nodes (A–F) and spleen (G–L) isolated from wild-type or IL7TG mice, untreated, treated with DMSO, or treated with **5H4** as indicated. Representative low power (A–C, G–I) and high power (D–F, J–L) fields are presented. One mm (low power) and 0.1 mm (high power) scale bars are embedded in the left image of each row. Lymph node follicular structures are indicated by black triangles and paracortical regions are indicated by black × marks (A–C). Spleen white pulp (stained blue) is indicated by black triangles and red pulp (stained red/purple) is indicated by black × marks (G–H).

mouse and one **5H4**-treated mouse died on day 1 and day 3 of the experiment. Those mice were replaced with previously untreated IL7TG mice and given the same amount of the treatment for the same period of time by extending their courses by 1 and 3 days, respectively. In the second experiment, only histology analysis was performed.

The lymph nodes of wild-type mice housed in specific-pathogen-free conditions are small and relatively quiescent (Figs. 5 and 6A and D). In contrast, the spontaneous lymphadenopathy of the IL7TG mice involves characteristic expansion of the follicular and paracortical regions containing abundant tightly packed monomorphic lymphocytes, seen as round cells darkly stained with hematoxylin (blue) and some effacement of structures (Fig. 6B and E). As shown in Figure 5, MRI measurements of the two analyzable mice that received 1 mg/day of compound **5H4** in DMSO for 14 days showed 30–50% reductions of lymph node volumes, while similar changes were not seen in the mice that received only DMSO. At necropsy, the lymph nodes of the **5H4**-treated mice appeared soft and deflated, in contrast to the turgid character of the lymph nodes of normal and DMSO-treated transgenic mice. Histological examination of lymph nodes of **5H4**-treated IL7TG mice revealed prominent spongy regions with fewer lymphocytes and a lightly eosin-stained (pink) stromal appearance (Fig. 6C and F), that is not observed in the lymph nodes from DMSO-treated IL7TG mice (Fig. 6B and E). Follicular structures were also reduced in size and cellularity in the lymph nodes of **5H4**-treated IL7TG mice (Fig. 6C). The IL7TG mice also exhibit splenomegaly with expanded regions of white pulp containing abundant lymphocytes and lymphocytic accumulations in the red pulp (Fig. 6G and J vs H and K). These expanded regions of white pulp were diminished in the spleens of **5H4**-treated IL7TG mice (Fig. 6). In

summary, the lymphadenopathy of IL7TG mice was markedly reduced by treatment with compound **5H4** and perturbations of the spleen architecture associated with the IL7TG were partially reversed.

3. Discussion

This study identified two novel derivatives of tyrphostin AG490, **5H4**, and **8B9**, which display significantly improved inhibition of the proliferation of cell lines that are dependent upon the continued activation of JAK2 or JAK3 kinases. Each of these molecules inhibit the proliferation of cytokine-dependent 2E8 cells and Baf/3 cells. They are substantially less effective as inhibitors of the factor-independent Jurkat acute T cell leukemia cell line and have minimal effects on the HL-60 acute promyelocytic leukemia cell line. Both **5H4** and **8B9** are trifluoromethyl derivatives of the parent compound tyrphostin AG490. These molecules each inhibit cytokine-induced JAK kinase activities and induce programmed cell death. To examine the in vivo efficacy of the most potent of these molecules, it was administered to lymphoma-prone IL7TG mice. Fourteen daily doses of 1 mg of **5H4** greatly reduced the volumes of the enlarged lymph nodes of these mice. Histological examination revealed significant reductions in lymph node cellularity.

The screening strategy employed in this study utilized cytokine-dependent cell lines to focus on signal transduction pathways. IL-7 signaling activates JAK3 and JAK1 kinases, whereas IL-3 signaling activates JAK2 kinase. Cell lines dependent upon these two parallel but distinct signal transduction pathways were used in an attempt to identify compounds with improved activity as well as any that might display differential activity. The trifluoromethyl derivatives **5H4** and **8B9** each inhibit both of these pathways. The Jurkat T cell leukemia cell line requires no exogenous factors for growth and is reported not to express JAK3.[33] Nonetheless, it is sensitive to the tyrphostins, but at higher concentrations than the factor-dependent lines. Even the non-lymphoid promyelocytic leukemia HL-60 cell line has some sensitivity to higher concentrations of **5H4**. Thus it appears that high concentrations of the tyrphostins may affect important molecules other than the JAK kinases.

AG490 has been shown to block the growth of acute lymphoblastic leukemia (ALL) cells by inducing programmed cell death.[31] Consistent with this, **5H4** and **8B9** also triggered programmed cell death in 2E8 and Baf/3 cells.

Among the 599 derivatives of AG490 in the combinatorial library, **5H4** and **8B9** were the most potent inhibitors of each of the factor-dependent cell lines and they were more effective inhibitors of Jurkat cells and HL-60 cells. They each have trifluoromethyl substitutions of the benzylamino group at either *meta* or *para* positions, respectively. The replacement of hydrogen atoms or hydroxyl groups by halogens or halogen-containing groups is a recognized strategy to alter, and in some cases improve, the biological function of drugs. It is noteworthy that a *para*-chloro substituted derivative was also more active than AG490 (data not shown). The trifluoromethyl group is lipophilic and thus it is likely to increase the extent to which the molecule is absorbed into cell membrane and can enhance the ability of the drug to pass through it.[48] It may also increase hydrophobic interactions between the drug and the target enzyme and might alter the metabolism of the molecule and extend its biological half-life.[49] While the cause of the improved activity of compound **5H4** is not addressed by this study, it appears most likely that it is due to an increased ability of the molecule to absorb into and pass through the cell membrane. Although AG490 and **5H4** are both likely to be susceptible to nucleophilic attack, there is no evidence that their biological activities involve chemical reactivity. The biological significance of the potential reactivity of these molecules is unclear.

It has recently been reported that a novel tyrosine kinase inhibitor CR4 ((*E*,*E*)-2-(benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrile) markedly inhibits JAK2 and Bcr-Abl kinase activity and the growth and survival of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).[50] CR4 differs from AG490 only in that the dihydroxystyryl group is displaced by two additional unsaturated carbon atoms in CR4. This study suggests that similar trifluoromethyl modifications of CR4 might also increase its potency.

Reduction of the spontaneous lymphadenopathy of IL7TG by treatment with tyrphostin **5H4** demonstrates that the trifluoromethyl modification does not interfere with the activity of the molecule in vivo. The ability of AG490 and its derivatives to induce apoptosis and the reductions in sizes of the lymph nodes that we observed in **5H4**-treated mice suggest the possibility of apoptosis. However, we failed to detect the presence of apoptotic cells using a TUNEL assay (data not shown). Nonetheless, it is possible that late stage apoptotic cells have been cleared from the tissue, since the overall lymph node volumes of **5H4**-treated mice decreased by 30–50%.

This study demonstrates a strategy of screening compounds for bioactivity using factor-dependent cell lines to focus on distinct signaling pathways. The improvement in biological activity associated with the addition of a trifluoromethyl group to the tyrphostin molecule might reflect an improved interaction with the cell membrane. The ability of **5H4** and other tyrphostins incorporating further modifications to impair JAK kinase signaling in vitro and in vivo may ultimately lead to their development as therapeutic agents.

4. Experimental

4.1. Cell cultures

The IL7-dependent murine pro-B cell line 2E8 (ATCC, TIB-239,[51]) was maintained in cDMEM medium

(Dulbecco's modified Eagle medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin, 10 mM Hepes, 1 mM sodium pyruvate, 1× non-essential amino acids, and 0.05 mM 2-mercaptoethanol), supplemented with 150 ng/mL of recombinant murine IL-7 (B. Rich and L. Cosenza, unpublished). The IL3-dependent murine pro-B cell line Baf/3[52,53] was maintained in RPMI-1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin, supplemented with 0.5 ng/mL of recombinant murine IL-3 (R&D Systems). Jurkat (ATCC, TIB-152) and HL-60 (ATCC, CCL-240) cells were maintained in RPMI-1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. All cells were incubated at 37 °C in 5% CO_2 and 100% humidity.

4.2. Chemicals

4.2.1. Combinatorial library. A chemical library of variants of AG490 was constructed in radio frequency-tagged reactors by a directed sorting method that incorporated variability at three sites within the molecule as described[45] (see Fig. 1B). Twenty different R^1 groups, 10 R^2 groups, and 3 R^3 groups were introduced to make a total complexity of 600 variants ($20 \times 10 \times 3 = 600$). One of the variants was lost because of a failed synthesis leaving 599 different compounds. Thirty of the compounds, selected at random were analyzed by electron spray mass spectrometry, ^{1}H NMR and gravimetric analysis as described[45] to confirm the accuracy of the syntheses (data not shown). Each of the 30 samples had molecular masses matching those predicted by the synthetic scheme. Twenty-one of the samples were greater than 80% pure; seven had purity between 50% and 80%; and two were less than 50% pure. The average yield of the syntheses was 15.2 ± 4.8 μmol. The specific precursors used for the syntheses are detailed in the Supplementary data. AG490 was purchased from Sigma and from A. Gazit, Hebrew University, Jerusalem.

4.2.2. *N*-(Cyanoacetyl)-(3-trifluoromethyl)benzylamide (3a). A mixture of methyl cyanoacetate (8.49 g, 85.6 mmol) and 3-(trifluoromethyl)benzylamine (15.0 g, 85.6 mmol) was heated at 100 °C for 6 h with no condenser. The resulting solid was triturated with 20 mL of 95% ethanol and the product filtered as a yellow solid (15.65 g, 75% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 3.43 (2H, s), 4.52 (2H, d, $J = 6$ Hz), 6.60 (1H, br s), 7.40–7.60 (4H, m).

4.2.3. 2-Propenamide, 2-cyano-3-(3,4-dihydroxyphenyl)-*N*-[[3-(trifluoromethyl)phenyl] methyl]-(*E*) (5H4) (NSC D722757). The amide **3a** from the previous reaction (5.0 g, 20.65 mmol), 3,4-dihydroxybenzaldehyde (2.76 g, 20.0 mmol), and piperidine (five drops) were refluxed in 95% ethanol (30 mL) for 1 h. The solution was cooled and 60 mL of water was added to precipitate the product. The solid was filtered, washed with 4×30 mL of water and evaporated under high vacuum to give 6.15 g (82% yield) of the desired product **5H4**, as a yellow solid. ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm) 4.67 (d, 2H, $J = 6.0$ Hz), 6.97 (d, 1H, $J = 8.4$ Hz), 7.38 (d, 1H, $J = 8.4$ Hz), 7.59–7.73 (m, 6H), 8.10 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm) 42.75, 100.08, 115.87, 117.13, 123.18, 123.82, 124.23, 128.75, 129.16, 129.64, 131.35, 131.83, 140.63, 145.69, 150.98, 151.23, 161.92. ES/MS m/z 361 $(M+H)^+$. Anal. Calcd for $C_{18}H_{13}F_3N_2O_3$: C, 59.67; H, 3.62; N, 7.73. Found: C, 59.59; H, 3.70; N, 7.76.

4.2.4. *N*-(Cyanoacetyl)-(4-trifluoromethyl)benzylamide (3b). A mixture of methyl cyanoacetate (5.66 g, 57.1 mmol) and 4-(trifluoromethyl)benzylamine (10.0 g, 57.1 mmol) was heated at 100 °C for 6 h with no condenser. The resulting solid was triturated with 12 mL of 95% ethanol and the product filtered as a yellow solid (11.0 g, 73% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 3.43 (2H, s), 4.53 (2H, d, $J = 6$ Hz), 6.60 (1H, br s), 7.40 (2H, d, $J = 8.1$ Hz), 7.62 (2H, d, $J = 8.1$ Hz).

4.2.5. 2-Propenamide, 2-cyano-3-(3,4-dihydroxyphenyl)-*N*-[[4-(trifluoromethyl)phenyl] methyl]-(*E*) (8B9) (NSC D722756). The amide **3b** from the previous reaction (6.0 g, 24.77 mmol), 3,4-dihydroxybenzaldehyde (3.31 g, 23.96 mmol), and piperidine (10 drops) were refluxed in 95% ethanol (36 mL) for 1 h. The solution was cooled and 72 mL of water was added to precipitate the product. The solid was filtered, washed with 4×40 mL of water and evaporated under high vacuum to give 6.70 g (75% yield) of the desired product **8B9**, as a yellow solid. ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm) 4.67 (d, 2H, $J = 6.0$ Hz), 6.97 (d, 1H, $J = 8.4$ Hz), 7.38 (d, 1H, $J = 8.4$ Hz), 7.59–7.73 (m, 6H), 8.10 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm) 42.75, 100.10, 115.88, 116.29, 117.18, 123.16, 124.93, 125.35, 125.64, 127.35, 127.74, 128.23, 144.02, 145.71, 151.02, 161.92. ES/MS m/z 361 $(M+H)^+$. Anal. Calcd for $C_{18}H_{13}F_3N_2O_3 \cdot 0.15 \cdot H_2O$: C, 59.23; H, 3.67; N, 7.67. Found: C, 59.05; H, 3.70; N, 7.90.

4.3. Screening and cell proliferation assays

Each of the compounds of the combinatorial library or AG490 were initially dissolved in DMSO at 1.5 mM. Subsequent dilutions were made in culture media. Ten microliters aliquots of appropriate dilutions were added to 100 μL cultures of factor-dependent 2E8 cells (100,000 per well) and Baf/3 cells (20,000 per well). After 48 h, 0.5 μCi of ^{3}H-thymidine (Perkin Elmer Life Sciences) in 25 μL of media was added to each well and the cultures were incubated for another 8 h. The cells were then lysed by hypotonic shock and DNA was captured onto glass fiber filters using an automated harvester (Tomtec). Incorporated ^{3}H-thymidine was quantitated by scintillation counting (Wallac, Model 1450 MicroBeta TriLux scintillation and luminescence counter). Statistical analysis was performed using GraphPad Prism software.

4.4. Flow cytometry analysis

Cells (1×10^6) were resuspended in 0.5 mL solution A (4 mM sodium citrate pH 7.8, 0.1% Triton X-100, 0.1

mg/mL RNase A, 50 μg/mL propidium iodide). After incubation at room temperature for 10 min, 0.5 mL of solution B (0.4 M sodium chloride, 0.1% Triton X-100, 50 μg /mL propidium iodide) was added and mixed well. Cells were stored at 4° for 2 h and subsequently subjected to fluorescent flow cytometry analysis with a Becton Dickinson FACScan machine. Data were collected from 1×10^5 cells. Images were generated and sub-G1 cell populations were counted using Cellquest software.

4.5. Immunoprecipitation and immunoblot analysis

Cells (1×10^6) were lysed in 0.5 mL lysis buffer [10 mM Tris–HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium *ortho*-vanadate, 1× protease inhibitor cocktail (Roche)] on ice, sonicated, and centrifuged at 10,000*g* for 10 min at 4°. Supernatant containing 500 μg protein was precleared with 20 μL of protein A/G plus-agarose beads (Santa Cruz) and then incubated with 5 μg of anti-JAK3 antibody (Upstate Biotechnology) and 20 μL of protein A/G plus-agarose beads at 4° overnight. The beads were washed three times with lysis buffer and separated on 4–20% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was probed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology), followed by goat anti-mouse IgG-HRP secondary antibody (Upstate Biotechnology). Complexes were detected by Super-Signal West Pico chemiluminescent substrate (Pierce Biotechnology).

4.6. Treatment of mice with 5H4

Age-matched groups of 12–16 week old IL7TG mice[8] with palpable lymphadenopathy were given daily intraperitoneal injections of **5H4** dissolved in 0.1 mL of DMSO or DMSO alone according to an institutional review board approved protocol.

4.7. Magnetic resonance imaging

Magnetic resonance images of wild-type and IL7TG mice were obtained by proton NMR scanning under inhaled isofluorane anesthesia with respiratory gating in an 8.5 T 9 cm vertical bore Bruker DRX magnet with a 3 cm diameter surface coil at 0.5 mm resolution with the support of the staff at the Center for Basic Magnetic Resonance Research of the Beth Israel Deaconess Medical Center.

MRI images were analyzed with Osiris imaging software (Digital Imaging Unit, Switzerland). The volumes of lymph nodes were calculated as the sums of the cross sectional areas of the organ from each slice in which it is visible, multiplied by the distance between slices (0.5 mm).

4.8. Histology

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Rodent Histopathology core facility of Dana-Farber/Harvard Cancer Center. Microscopic images were captured with a Spot camera (Diagnostic Instruments, Inc.) using a Nikon Eclipse E600 microscope (Nikon Corporation) and processed with Spot 3.5.5 software (Diagnostic Instruments, Inc.).

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Supplementary data

Three files showing the precursors used in the combinatorial synthesis are provided as supplementary files. Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.bmc.2005.04.022.

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