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Photochemical Preparation of a Pyridone Containing Tetracycle: A Jak Protein Kinase Inhibitor

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Abstract—Jak3 is a protein tyrosine kinase that is associated with the shared γ chain of receptors for cytokines IL2, IL4, IL7, IL9, and IL13. We have discovered that a pyridone-containing tetracycle (**6**) may be prepared from trisubstituted imidazole (**5**) in high yield by irradiation with >350 nm light. Compound **6** inhibits Jak3 with $K_I = 5$ nM; it also inhibits Jak family members Tyk2 and Jak2 with $IC_{50} = 1$ nM and murine Jak1with $IC_{50} = 15$ nM. Compound **6** was tested as an inhibitor of 21 other protein kinases; it inhibited these kinases with IC_{50}s ranging from 130 nM to >10 μM. Compound **6** also blocks IL2 and IL4 dependent proliferation of CTLL cells and inhibits the phosphorylation of STAT5 (an in vivo substrate of the Jak family) as measured by Western blotting.

Janus protein tyrosine kinases (Jaks) associate with the intracellular portions of many cytokine and growth hormone receptors.[1,2] A Jak family protein kinase is characterized by seven regions of sequence homology[3] and four family members have been identified to date. These are Jak1, Jak2, Jak3, and Tyk2. Jak1, Jak2, and Tyk2 are expressed almost ubiquitously whereas Jak3 appears to be expressed principally in hematopoietic cells. Jaks transduce extracellular signals by phosphorylating cytoplasmic proteins, among the best characterized of which are the signal transducers and activators of transcription (STATs). The STATs dimerize on phosphorylation and directly activate transcription after nuclear translocation.[3] These signaling events mediate such diverse biological outcomes as thymocyte development and erythrocyte differentiation.[4] Congenital Jak3 deficiency is associated with an immune compromised (SCID) phenotype in both rodents[4] and humans.[5,6] The SCID phenotype of Jak3 −/− mammals and the lymphoid cell specific expression of Jak3 are two favorable attributes of a target of an immune suppressant.

Tyrosine kinase researchers have generated inhibitors in a variety of structural classes.[7] Previous literature reports of Jak family inhibitor scaffolds with efficacy in mouse disease models have included benzylidenemalonitriles (tyrphostins)[8,9] and quinazolines (WHI-P154, and WHI-P151).[10,11] Meydan showed that AG-490, a tyrphostin Jak2 inhibitor, could inhibit the proliferation of leukemic cells from acute lymphoblastic leukemia patients.[12] No dissociation constants measured by steady state kinetic methods were reported for these inhibitors but both the tyrphostins and WHI-P151 inhibit Jak driven biological events at micromolar concentrations.[8,10]

We report here the synthesis, biochemistry, and cellular activity of **6** (Scheme 1), a pyridone-containing tetracycle that inhibits the Jak family of protein tyrosine kinases and Jak driven signaling events at concentrations 100-fold lower than those required for inhibition by compounds based on tyrphostin or quinazoline scaffolds. 2,4,5-Trisubstituted imidazole **5** was prepared according to Scheme 1.[13] Regioisomerically pure pyridone **6** was found to form nearly quantitatively after 45 min of irradiation of a 3.2 mM solution of **5** in THF with light of wavelength greater than 350 nm at 1 mW

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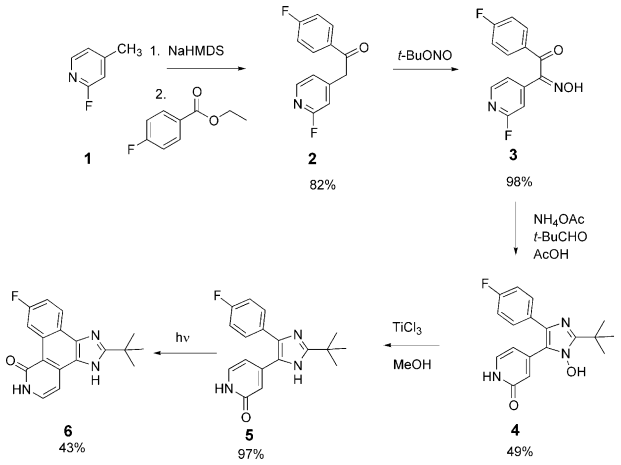

Scheme 1. Synthesis of pyridone **6**.

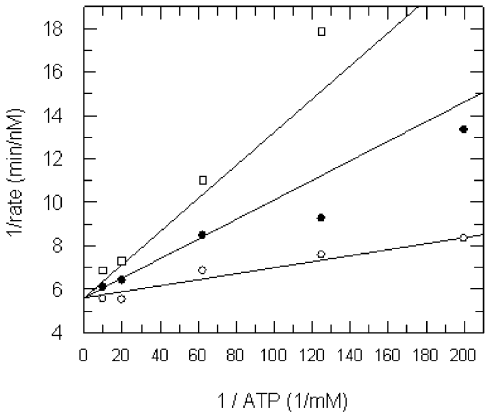

Figure 1. Pyridone **6** is competitive with ATP. ATP was varied as a function of [**6**]; open circles, [**6**] = 0 nM, [**6**] = 5 nM, [**6**] = 10 nM. The data were fit to rate = $(V_{max}\times[ATP])/([ATP]+K_m^{ATP}\times(1+[\mathbf{6}]/K_I))$ which describes competitive inhibition. Parameters from the fit are $V_{max} = 0.18\pm0.01$; $K_m^{ATP} = 2.5\pm0.5$ μM; $K_I = 2.2\pm0.6$ nM.

intensity.[15] Although the conversion of **5** to **6** was >95% by HPLC (not shown) recrystalization of **6** lowered the final yield to 43%.[14] 2,4,5-Trisubstituted imidazoles have been known to be protein kinase inhibitors since Lee demonstrated that the target of antiinflammatory 2,4-diaryl-5-pyridin-4-yl-imidazoles was the serine/threonine kinase p38.[15] Photochemically driven oxidative cyclization of 4,5-diphenylimidazole and other similar compounds has also been reported; the reactions required long irradiations (4–20 h) with a 125 W source.[16] However, at the time of writing, no reports of the properties of cyclized trisubstituted imidazoles as protein kinase inhibitors have appeared in the literature.

The photochemistry of 2-pyridones has been explored extensively. 1,4-Bicyclic 'photopyridone' has been isolated and characterized.[17] Irradiation of 2-pyridones can also yield [4 + 4] dimers, a reaction useful both for intra- and inter-molecular conversions[18,19] but these reactions do not typically result in oxidized products. Orienting the photoactivatable pyridone in a trisubstituted imidazole such that photocyclization may occur yields a molecule **5** that undergoes nearly quantitative oxidative photocyclization to form **6**. Moreover, the pyridone facilitates the reaction relative to other substituents. For instance, no cyclization occurred following replacement of the pyridone of **5** with a pyridinyl moiety using irradiation conditions that completely converted **5** to **6** (data not shown).

Based on analysis of the draft human genome sequence Venter[20] estimates there are 868 human kinases and attaining sufficient specificity remains a formidable obstacle to therapeutic deployment of protein kinase inhibitors.[7] Steady-state kinetic studies show that **6** is a reversible inhibitor that is competitive with respect to ATP (Fig. 1) and non-competitive with respect to peptide substrate (data not shown). This occurs because, like many protein kinase inhibitors,[7] **6** shares binding determinants with adenosine, a ligand ubiquitous in living systems. Although **6** binds in a site common to all protein kinases it shows specificity for the Jak family of protein tyrosine kinases over other kinases and limited selectivity within the family. Tyk2 and Jak2 ($IC_{50} = 1$ nM) are inhibited slightly more potently than Jak3 and Jak1 ($IC_{50} = 5$ and 15 nM, respectively). The dual specificity kinase Mek is inhibited about 30-fold less potently than Jak3, and the other tested kinases are all inhibited more weakly (Table 1).

CTLL cells (a murine T cell lymphoma cell line) can be induced to proliferate with the cytokines IL2 or IL4, whose receptors have both Jak1 and Jak3 associated with the intracellular domains.[21] The tetracyclic pyridone **6** inhibits IL2 driven proliferation of CTLL cells with $IC_{50} = 0.1$ μM and IL4 driven proliferation with $IC_{50} = 0.052$ μM (Table 2). Phorbol myristate acetate is a phorbol ester that does not require Jak family signaling to stimulate proliferation,[22,23] so inhibition of PMA driven proliferation is a measure of specificity within a

Table 1. Inhibition of protein kinase activity[26] by **6**

Kinase	IC_{50} (μM)
Jak3	0.005
Tyk2	0.001
Murine Jak1	0.015
Jak2	0.001
Cyclin-dependent kinase 2	3.3
cAMP-dependent kinase	7.1
PKC(α)	1.2
Csk	2.1
Hck	7.7
Lck	>8
Fyn T	0.5
Lyn	>8
src	>8
p38	11
MAPK	1.78
Mek	0.16
Raf	>10
IκB Kinase 2	0.30
KDR	1.4
Flt-1	1.52
Flt-4	0.69
FGFR	1.48
FGFR2	0.94
Tek	24
PDGFR	1.49

Table 2. Inhibition of Proliferation of CTLL cells[27] by pyridone **6**

Stimulus of proliferation	IC_{50} of **6** (μM)
IL2	0.10
IL4	0.052
PMA	3.1

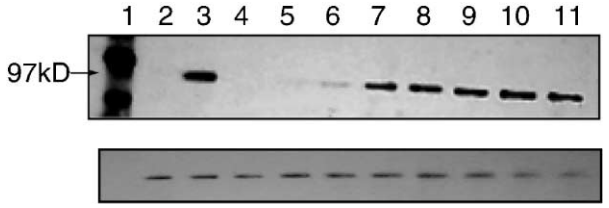

Figure 2. Inhibition of STAT5 phosphorylation in IL2 stimulated CTLL cells by **6** as measured by Western blotting of cellular proteins with an antibody to phosphorylated STAT5. The top row of lanes show lysates from cells were treated as follows: Lane 1: molecular weight markers; lane 2: No IL2 added; lane 3: IL2 only; lane 4: IL2 + 600 nM **6**; lane 5: IL2 + 200 nM **6**; lane 6: IL2 + 67 nM **6**; lane 7: IL2 + 22 nM **6**; lane 8: IL2 + 7 nM **6**; lane 9: IL2 + 2 nM **6**; lane 10: IL2 + 0.67 nM **6**; lane 11: IL2 + 0.22 nM **6**. The bottom row shows the same samples analyzed by Western blotting with an antibody to STAT5.

cellular environment. Pyridone **6** inhibits PMA induced proliferation, but with $IC_{50} = 3.1$ μM (Table 2). Accordingly, we attribute these effects of **6** at these concentrations to inhibition of non-Jak family protein kinases, although a more general cellular toxicity cannot be ruled out.

Jaks phosphorylate STATs in response to extracellular signals.[3] In the case of IL2 stimulation, this event may be followed using Western blotting with an antibody that is specific for the phosphorylated form of STAT5. The estimated IC_{50} (22 nM, Fig. 2) for inhibition of STAT5 phosphorylation by **6** is qualitatively in agreement with the results for inhibition of proliferation (100 nM, Table 2). It has been shown that STAT5a or b is required for a variety of growth hormone responses,[24] and splenocytes from STAT5a/5b knockout mice show diminished responsiveness to IL2 and IL4.[25] The proportional inhibition of STAT5 phosphorylation and proliferation implies that STAT5 phosphorylation is rate-limiting for the proliferative response in CTLL cells, at least at a population level.

Considered together, these data paint a consistent picture of **6** action via Jak kinase inhibition. It is an inhibitor of Jak3 with $IC_{50} = 5$ nM and Jak1 with $IC_{50} = 15$ nM (Table 1). The effect of this inhibition on IL2 driven CTLL cell proliferation is shifted upward to $IC_{50} = 100$ nM (Table 2), and a measure of proximal signaling (STAT5a/b phosphorylation) is concomitantly inhibited (Fig. 1). The nonspecific effects of **6** are evident in the inhibition of PMA induced cell proliferation but the IC_{50} is considerably higher. It remains to be seen if selective Jak3 inhibition by tetracylic pyridones will block IL2 induced T cell activation as effectively as the Jak family inhibitor **6**.

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13. **2-[4-(2-Fluoropyrididyl)]-1-(4-fluoro)phenylethan-1-one** (**2**). To sodium bis(trimethylsilyl)amide (0.465 mol, 1 M) in THF (765 mL) under nitrogen, cooled to 2 °C, was added 2-fluoro-4-methylpyridine (25 g, 0.225 mol) and the solution aged for 45 min in an ice bath. Ethyl 4-fluorobenzoate (35 mL, 0.239 mol) was added and the reaction aged for 1.5 h. The reaction was poured into excess aqueous 2 N HCl. The aqueous layer was made basic with 5 N NaOH and extracted with ethyl acetate. The ethyl acetate extracts were combined, washed with saturated NaCl, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and filtered through a cotton plug. Hexanes were added and the CH_2Cl_2 removed under reduced pressure until precipitation of **2** occurred as a pale-yellow solid (43.2 g, 82%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.19 (br s, 1H), 8.04 (s, 2H), 7.19 (m, 2H), 7.09 (s, 1H), 6.86 (s, 1H), 4.32 (s, 2H). MS: (*m*/*z* 234.2 (M + 1)). $t_R = 2.81$ min, YMC C18-S-5 column, 10/90→95/5 MeCN/H_2O (0.05% TFA) linear gradient, 2.5 mL/min.

2-Hydroximino-2-[4-(2-fluoropyrididyl)]-1-(4-fluoro)phenylethan-1-one (**3**). To **2** (43 g, 0.184 mol) was added ethanol (800 mL) and cooled under nitrogen to −10 °C using a dry ice/isopropanol bath. To this mixture was added dropwise *t*-butylnitrite (24.1 mL, 0.20 mol) over 10 min, followed by 2.5 M HCl in absolute ethanol (60 mL, 0.15 mol). The reaction temperature was maintained at −5 °C during these additions. After addition was complete, the dry ice bath was removed and the reaction was allowed to warm and aged overnight. The ethanol was removed under reduced pressure and the solution was diluted into H_2O and made basic with saturated $NaHCO_3$. The solution was extracted with ethyl acetate, the combined organic fractions were washed with saturated NaCl, dried over Na_2SO4 and concentrated under reduced pressure to yield crude product **3**. Crude **3** was taken up in methanol/isopropanol and mixed with toluene. The methanol/isopropanol mixture was concentrated under reduced pressure and **3** was recrystallized from hexane/toluene (47.5 g, 98% yield). ^{1}H NMR (CD_3OD, 500 MHz) δ 7.95–8.27 (m, 3H), 7.10–7.47 (m, 4H). MS: (*m*/*z* 263.1 (M + 1). $t_R = 2.83$ min, YMC C18-S-5 column, 10/90→95/5 MeCN/H_2O (0.05% TFA) linear gradient, 2.5 mL/min.

2-(1,1-Dimethylethyl)-5-(4-fluorophenyl)-3-*N*-hydroxy-4-(pyrid-3-onyl)-imidazole (4). To **3** (47.3 g, 0.18 mol) in acetic acid (1 L) under nitrogen was added trimethylacetaldehyde (21.6 mL, 0.19 mol) followed by ammonium acetate (277.5 g, 3.6 mol) and then heated to reflux for 3 h. The acetic acid was removed under reduced pressure and the remaining material slurried in water. The pH of the solution was adjusted to 8–10 by addition of solid ammonium hydroxide and extracted with ethyl acetate. The volatiles were removed under reduced pressure and the crude product **4** dissolved in methanol with sufficient ethanol added to effect dissolution. The volatiles were removed under reduced pressure; the product **4** was twice dissolved in ethanol and concentrated to a small volume under reduced pressure to azeotropically remove water. Tricycle **4** was recrystallized from ethanol and hexane (28.8g, 49%).[1]H NMR (DMSO-d_6, 500 MHz) δ 11.55 (s, 1H), 11.34 (s, 1H), 7.45 (m, 2H), 7.32 (s, 1H), 7.09 (m, 2H), 6.41 (br s, 1H), 6.02 (br s, 1H), 1.37 (s, 9H). MS: (m/z 328.1 (M + 1)). t_R = 1.57 min, YMC C18-S-5 column, 10/90→95/5 MeCN/H_2O (0.05% TFA) linear gradient, 2.5 mL/min.

2-(1,1-dimethylethyl)-9-fluoro-1*H*-benz[*h*]isoquinolin-3-one-[5,6-d]imidazole (5). To **4** (28 g, 0.086 mol) in methanol (I L) at 0 °C under nitrogen was added Ti (III) Cl_3 (10% w/w in 20% w/w HCl, 450 mL) over 45 min while maintaining the reaction temperature under 10 °C. The solution was warmed and aged overnight. The methanol was removed under reduced pressure and the solution was made basic with saturated $NaHCO_3$ and 5 N NaOH. Ethyl acetate was added and the reaction aged for 4 h. The solution was filtered through a Solka floc pad to remove the solids. The filtrate was extracted with ethyl acetate and the organic layer was then washed twice with saturated NaCl, dried over Na_2SO_4 and concentrated to dryness to yield crude **5**. The crude **5** was purified by flash chromatography using CH_2Cl_2/2–10% methanol as an eluant to yield pure **5** (26 g, 97%). [1]H NMR [DMSO-d_6, 500 MHz, mixture of imidazole tautomers (~2:1)] δ 12.05 (s, 2/3H), 11.91 (s, 1/3H), 11.25 (br s, 1H), 7.47 (m, 2H), 7.28 (m, 2H), 7.17 (m, 1H), 6.43 (d, J = 1.4 Hz, 1/3H), 6.32 (d, J = 1.4 Hz, 2/3H), 6.23 (dd, J_1 = 6.8 Hz, J_2 = 1.8 Hz, 2/3H), 5.98 (dd, J_1 = 6.8 Hz, J_2 = 1.8 Hz, 1/3H), 1.33 (s, 9H). MS: (m/z 312.2 (M + 1)). t_R = 1.49 min, YMC C18-S-5 column, 10/90→95/5 MeCN/H_2O (0.05% TFA) linear gradient, 2.5 mL/min.

14. **2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7*H*-benz[*h*]-imidaz[4,5-*f*]isoquinolin-7-one (6)**. To **5** (2.0 g, 6.4 mmol) in a Pyrex vessel was added THF (2 L) and the solution was irradiated with stirring in a Pyrex vessel with light >350 nm for 45 min. During irradiation, the solution transiently turned a reddish pink; the color disappeared on further stirring without irradiation. At this juncture, further irradiation did not reinitiate the reaction. Two more batches of **5** (2.0 g, 6.4 mmol) were treated identically. The solvent was removed under reduced pressure to yield crude **6**. The crude material was purified by flash chromatography on silica gel using THF/toluene (3/7→7/3) as an eluant. Pure **6** (2.6 g, 43%) was obtained following recrystallization from methanol. [1]H NMR [DMSO-d_6, 500 MHz, mixture of imidazole tautomers (~2:3)] δ 12.84 (s, 3/5H), 12.67 (s, 2/5H), 11.67 (s, 2/5H), 11.59 (s, 3/5H), 10.05 (m, 1H), 8.55 (m, 1H), 7.60 (m, 1H), 7.51 (br s, 1H), 7.31 (br s, 2/5H), 7.25 (br s, 3/5H), 3.32 (s, 9H). MS: (m/z 310.2 (M + 1)). t_R = 1.81 min, YMC C18-S-5 column, 10/90→95/5 MeCN/H_2O (0.05% TFA) linear gradient, 2.5 mL/min.

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26. **Assay for Jak family protein kinase activity.** Jak3, Tyk2, Jak1 and Jak2 kinase domains with N-terminal 'Flag' affinity tags were expressed in Sf9 cells using standard baculovirus methods and purified by antiFlag affinity chromatography, followed by gel filtration chromatography. This yielded soluble monomeric Jak family kinase at >90% purity by SDS-PAGE (not shown). The human Jak3 gene was provided by Dr. John J. O'Shea (NIH). The human Tyk2 gene was provided by Dr. Sandra Pellegrini (Insitut Pasteur). Human Jak2 kinase domain was cloned from a MOLT4 cDNA library (Clonetech). Murine Jak1 was cloned from a spleen cDNA library (Clonetech). Jak family tyrosine kinase activity was measured by detection of the tyrosine phosphorylated peptide amino hexanoyl biotin-EQEDEPEGDYFEWLE-NH2 (S, hereafter) detected by homogenous time resolved fluorescence (HTRF) using a europium labeled antibody to phosphotyrosine (pY20).[28] For determining the mode of inhibition of **6** with respect to peptide substrate, γ^{33}P ATP was included in the assays and incorporated radioactivity was measured using SAM^2 (Promega, Madison, WI, USA). **Assay methods for PKA, PKCα and cdk2.** Cyclin E•Cyclin dependent kinase 2 (cdk2) complex was provided by Dr. Charles Omer (Merck Research Labs) and was assayed by SPA using the biotinylated peptide PKTPKKAKKL from Promega. Bovine PKA catalytic subunit (Promega) was assayed by SPA using the biotinylated peptide LRRASLG (Promega) as a substrate. PKCα (Upstate Biotechnology) was assayed by SPA using the biotinylated peptide AAKIQASFRGHMARKK (Promega). Streptavidin coated SPA beads and γ^{33}P ATP were from Amersham Pharmacia Biotechnology. **Raf, Mek, MAPK assay methods**. Raf, MAPK, GST-kinase dead MAPK, and GST-kinase dead Mek, were provided by Dr. Hans Huber (Merck Research Labs). Bovine myelin basic protein (MBP) and active Mek kinase were from Upstate Biotechnology. The enzyme/substrate pairs used were Raf with GST-kinase dead Mek, Mek with kinase dead MAPK, and MAPK with MBP all in the presence of γ^{33}P ATP (Amersham Pharmacia). Reactions were applied to PVDF filter plates (Millipore) and the captured radioactive product measured by scintillation counting. **Assay methods for remaining kinases.** Csk, Hck, Lck, FynT, Lyn and src were assayed as described previously.[28] IκB Kinase 2[29] and p38[30] were assayed as described previously. KDR, Flt-1, Flt-4, FGFR, FGFR2, Tek, and PDGFR were assayed as described previously.[31]

27. **Cellular proliferation assays.** CTLL-2 cells (ATCC) were maintained in 6% T-stim Culture Supplement (source of IL2) in RPMI-1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 1.4 mM L-glutamine, 10 mM HEPES, 1 mg/mL dextrose, 0.04 mM essential amino acids, 0.02 mM nonessential amino acids, penicillin and streptomycin (H10). The day before use in the proliferation assay, cells were washed and resuspended in 0.2% Tstim at a cell concentration of 5×10^5/mL. The next day, cells were washed and plated at $0.2–1\times10^5$ cells/well in a 96-well tissue culture plate (CoStar). 0.05 ng/mL mouse recombinant IL2 (Pharmingen) or 20 ng/mL PMA (Sigma) and 1 μCi/well [^{3}H]-thymidine were added. After overnight culture, cells were harvested with a glass fiber Filtermat (Wallac) and a Tomtek cell harvester. Tritium incorporation was measured by liquid scintillation counting on a Topcount scintillation counter (Packard).

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