Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2489–2494

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THE DEVELOPMENT OF NOVEL AND SELECTIVE p56^{lck} TYROSINE KINASE INHIBITORS[1]

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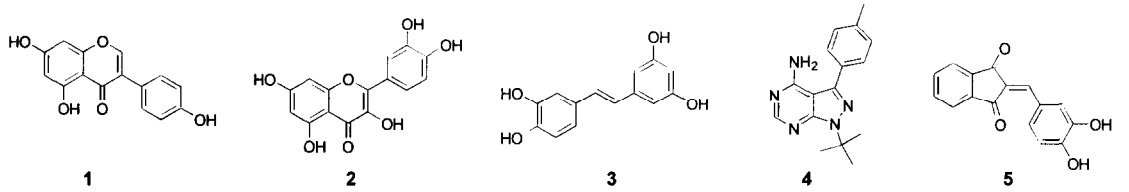
Received 22 May 1998; accepted 27 July 1998

Abstract: Early T-cell receptor mediated signal transduction involves the activation of several tyrosine protein kinases. One of these tyrosine kinases, p56^{lck}, is expressed primarily in T-cells and Natural Killer (NK) cells and has been shown to be critical for their proliferative and effector functions. Indandiones have been identified as a potent and selective chemical class that inhibits p56^{lck}.

Early T-cell receptor mediated signal transduction involves the activation of several protein tyrosine kinases.[2] One of these tyrosine kinases, a src family tyrosine kinase, p56^{lck},[3-5] is expressed primarily in T-cells and Natural Killer (NK) cells.[6] p56^{lck} kinase has been shown to be critical for T-cell proliferative and effector functions.[7] Because p56^{lck} kinase is found mainly in T-cells and NK cells, a selective inhibitor has the potential to suppress the immune response with minimal toxic effects.

Many compounds reported to inhibit p56^{lck} kinase show similar features.[8-10] Inhibitors of tyrosine kinases have been generally shown to be flat aromatic structures, usually containing electron donor and electron acceptor groups separated by an aromatic spacer thus creating an electronic push-pull situation across the molecule.[11] Often these inhibitors are highly oxygenated species represented by the polyphenols, Genistein, (**1**)[12,13] Quercetin, (**2**)[14,15] and Piceatannol (**3**).[16] Because of the relatively high homology among tyrosine kinases, many of the known inhibitors lack the specificity necessary to avoid effects outside of the immune system, although family-specific tyrosine kinase inhibitors such as compound **4** have been reported.[17] We postulated that binding to a specific tyrosine kinase could be enhanced by the optimization of the stereoelectronic characteristics of the molecule. We based our discovery efforts on this hypothesis and prepared compounds that exhibited flat, conjugated phenolic structures which could be substituted with electron donating and electron withdrawing groups. Compound **5** quickly emerged as an initial lead having an IC_{50} of 5 μM against p56^{lck}.

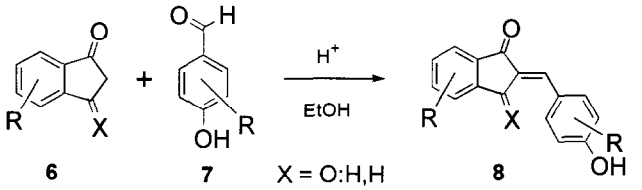
0960-894X/98/$ - see front matter
PII: S0960-894X(98)00445-4



1 2 3 4 5

In order to explore the SAR of this chemical series and further elucidate the pharmacophore for $p56^{lck}$ activity, we prepared a number of different substituents on both the indanone (X = H,H)/indandione (X = O) portion of the molecule and also the benzylidene portion by condensing the respective indanone or indandione **6** with a variety of benzaldehydes **7** to give the targeted compounds **8** (Scheme 1).[18,19]

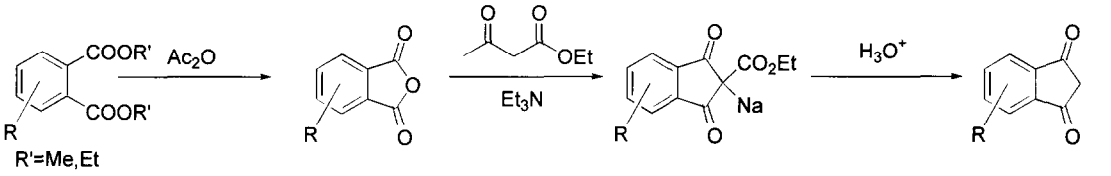
Scheme 1



6 7 X = O:H,H 8

A number of substituted indandiones were prepared following previously published procedures usually involving the condensation of substituted phthalic acid esters **9** to give the anhydrides **10** followed by Knoevenagel condensation with ethylacetoacetate. The resultant sodium salt of ester **11** could be decarboxylated to give the indandiones **12**. Alternatively, **11** could be used directly to condense with aldehydes to give the targeted compounds[20] (Scheme 2).

Scheme 2



R'=Me,Et

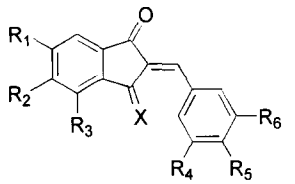
9 10 11 12

The compounds were assayed in vitro for their ability to inhibit $p56^{lck}$.[21] Inhibition of $p56^{lck}$ is dependent upon the electronic characteristics of both the indandione system and the benzylidene moiety. Some of our findings are presented in Table 1. Our data have shown that electron-withdrawing groups substituted on the indandione system generally caused a decrease in the inhibition of the enzyme compared to the unsubstituted parent molecule. For instance, compound **17**, with a nitro group substituted at the 5-position of the indandione is significantly less potent than the unsubstituted parent, compound **15**. Halogen substitution of the indandione system provided no significant change in potency when compared to the unsubstituted parent, compound **15** vs.

16 for example. Electron donating groups have been shown to generally cause an increase in inhibition relative to the unsubstituted parent molecules. Substitution with a methoxy group at the 5-position of the indandione increased potency five-fold when compared to the parent (compound **18** vs. **15**). A 5,6-dimethoxy substitution pattern showed no significant increase in potency compared to the 5-methoxy substitution (compound **27** vs.

Table 1

$p56^{lck}$ inhibition by substituted indanones/indandiones



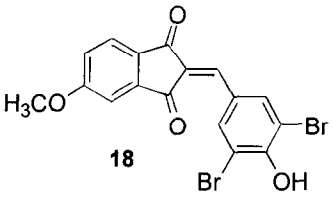
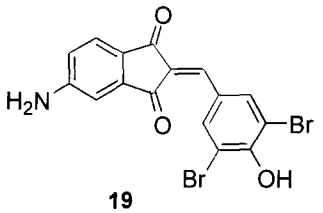
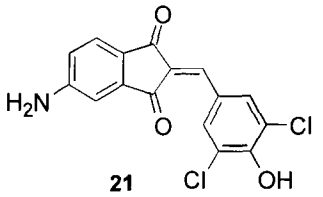
Compd	R_1	R_2	R_3	R_4	R_5	R_6	X	IC_{50} (μM)
3	H	H	H	H	OH	OH	O	5.0
13	NO_2	H	H	H	OH	OH	O	>5
14	NH_2	H	H	H	OH	OH	O	0.60
15	H	H	H	Br	OH	Br	O	0.74
16	Br	H	H	Br	OH	Br	O	1.0
17	NO_2	H	H	Br	OH	Br	O	>5
18	OMe	H	H	Br	OH	Br	O	0.14
19	NH_2	H	H	Br	OH	Br	O	0.032
20	NH_2	H	H	OMe	OH	OMe	O	1.7
21	NH_2	H	H	Cl	OH	Cl	O	0.10
22	OMe	OMe	H	Cl	OH	Cl	O	0.31
23	NH_2	H	H	Br	OH	Br	H,H	0.71
24	H	NH_2	H	Br	OH	Br	H,H	5.0
25	OMe	H	H	H	OH	OH	O	1.5
26	OMe	H	H	Br	OH	OMe	O	0.88
27	OMe	OMe	H	Br	OH	Br	O	0.11
28	Cl	Cl	H	H	OH	OMe	O	5.0
29	Cl	Cl	H	Br	OH	OMe	O	1.7
30	H	H	NO_2	H	OH	OH	O	5.0
31	H	H	NH_2	Br	OH	Br	O	0.47
32	H	H	NO_2	Cl	OH	Cl	O	1.4

18). An amino group provided the greatest increase in potency, resulting in a > 20 fold increase in inhibition over the unsubstituted analog (compound **19** vs. **15**). A 4-hydroxy group on the benzylidene portion of the molecule is critical for activity, which compares with the known $p56^{lck}$ inhibitors **1**, **2**, and **3**. Indandiones are

significantly more potent than indanones (compounds **23** and **24** vs. **19**). The 3,5-dibromo-4-hydroxy substituted benzylidene is optimal over other substitution patterns prepared including other 3,5-dihalo-4-hydroxy substitutions (compound **19** vs. **21**). Cushman et. al. have also seen increased activity in their $p56^{lck}$ inhibitors with this substitution pattern.[22] The most potent compounds in the series were evaluated in other kinase assays to determine their enzyme selectivity. Inhibition studies were run using the related tyrosine kinase c-src[21] and Protein Kinase A[23] (PKA). The results are summarized in Table 2.

Table 2

Selectivity data of most potent compounds

	Enzyme Inhibition IC_{50} μM		
	Lck	c-Src	PKA
 **18**	0.144	>50	10
 **19**	0.032	0.46	8
 **21**	0.102	3.5	5

As Table 2 indicates, our three most potent compounds are selective for $p56^{lck}$ kinase with significantly reduced activity towards the related kinase c-src and also PKA. The 5-aminoindandiones **19** and **21** were greater than 50 times less potent in the PKA assay while showing at least 14 times less activity against c-src. The 5-methoxyindandione **18** again showed good selectivity for $p56^{lck}$ over PKA. At concentrations above 0.4 μM **18** appears to stimulate c-src activity. Compounds **18**, **19**, and **21** were concluded to be ATP competitive, based on

double reciprocal plots of $1/v_o$ vs. 1/[S] (Lineweaver-Burke plots). These plots generated a series of straight lines intersecting a common point on the y axis ($1/v_o$), and were therefore concluded to be competitive for the ATP substrate.

In summary, we have identified a novel series of indandiones that exhibit potent inhibition of $p56^{lck}$ in vitro. Using our initial electron donor/acceptor hypothesis that the optimization of electron donating and electron withdrawing groups substituted on a flat, conjugated phenolic system could enhance the binding of these compounds to a specific tyrosine kinase, we have produced a greater than 1000-fold increase in potency over our earliest leads resulting in compounds that inhibit $p56^{lck}$ in the nanomolar range. The indandione series has been shown to be selective for $p56^{lck}$ over the related kinase c-src and PKA generally having at least 14 times greater inhibition of $p56^{lck}$ over c-src and at least 50 times greater inhibition over PKA. Encouraged by the potency and selectivity of this series, we are continuing to evaluate the series for selectivity against other enzymes, as well as for in vivo activity.

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19. **Representative procedure for indandione condensation: 2-[(3,5-Dibromo-4-hydroxyphenyl)methylene]-1H-1,3(2H)-dione 15:** 1,3-Indandione (0.70 g, 4.79 mmol) and 3,5-Dibromo-4-hydroxybenzaldehyde was dissolved in abs. ethanol (30 ml). The mixture was saturated with gaseous hydrogen chloride and heated to reflux for 18 h. The resulting slurry was cooled to room temperature, filtered, and washed with abs. ethanol to give **15** (1.74 g, 89%) as a light brown solid. **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.91 (2H, s), 7.95 (4H, m), 7.73 (1H, s). mp 329-331 °C

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21. Compounds were assayed for their effect on lck and c-src activity using an in vitro kinase assay where phosphate from ATP (50 μM) was transferred to tyrosine residues on a biotinylated peptide of p34 cdc-2. Quantitation of substrate phosphorylation via an ELISA-based colorimetric detection system involved binding an aliquot of the stopped kinase reaction to an avidin-coated plate followed by incubation with a peroxidase-conjugated monoclonal antibody specific for phosphotyrosine. The presence of an enzymatic inhibitor decreased substrate phosphorylation as measured by decreased light absorbance in the ELISA relative to an uninhibited control.

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23. Compounds were assayed for their effect on PKA activity utilizing γ-^{33}P-ATP to tag phosphorylated serine residues on a peptide substrate (Kemptide). After terminating the reaction, the substrate was filtered and retained on a phosphocellulose disk, taking advantage of charge interactions between basic residues of the peptide and the filter. Washed filters were counted and specific incorporation was calculated relative to a background control (absence of enzyme). Inhibition of enzyme activity by test compounds was detected as a decrease in cpm relative to the uninhibited control.