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The Design, Synthesis and Activity of Non-ATP Competitive Inhibitors of pp60$^{c\text{-}src}$ Tyrosine Kinase. Part 1: Hydroxynaphthalene Derivatives

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Abstract—A series of hydroxynaphthalene pp60$^{c\text{-}src}$ non-peptide inhibitors was designed, using the crystal structure of the insulin receptor tyrosine kinase as a qualitative model, to target the peptide substrate binding site. Representative inhibitors were shown to bind non-competitively with respect to ATP.

Protein tyrosine kinases (PTKs) are signal transduction enzymes that have been shown to be extensively involved in the formation and maintenance of cancer. Consequently, an intense effort is currently underway to develop non-peptide inhibitors for individual PTKs,[1] some of which have advanced to clinical trials. pp60$^{c\text{-}src}$ is a prototype for the nonreceptor PTK's and is an emerging target for drugs that may be useful in treating a wide range of cancers.[2,3] A knock-out of the '*src*' gene in mice led to only one defect; osteoclasts that do not resorb bone. However, this lone defect can be prevented by inserting a *src* gene that produces mutant pp60$^{c\text{-}src}$ wherein the enzymatic kinase activity has been lost.[4] These and other reports suggest that inhibition of pp60$^{c\text{-}src}$ could provide broad anti-cancer activities without inducing serious toxicity.

The vast majority of known PTK non-peptide inhibitors bind in the highly conserved ATP substrate site.[1] Nevertheless, some of these ATP competitive PTK inhibitors have been shown to have good selectivity when tested against a small panel of isolated protein kinases. However, it is estimated that there are approximately 2000 different protein kinases (in addition to other ATP-utilizing proteins) in humans,[5] all of which utilize ATP as the second substrate. Testing a given inhibitor against a full panel of isolated kinases is currently not feasible. Consequently, targeting PTK inhibitors for the more unique peptide substrate binding sites can provide a greater level of confidence that acceptable selectivity has been achieved. A second potential advantage of this alternate approach is that inhibitors binding ca. 1000-fold weaker than ATP-competitive inhibitors could be equally potent in the intracellular environment wherein they may be competing with μM concentrations of external protein substrates rather than mM levels of ATP.[1]

The utilization of naphthalene-based peptide substrate mimics as non-ATP competitive PTK inhibitors was first introduced by Saperstein et al.[6] in their rational design of non-peptide insulin receptor (IRTK) and epidermal growth factor receptor (EGFRTK) PTK inhibitors.[6] This concept was later extended into the analogous quinoline, isoquinoline, and iminochromene rings by Burke et al.[7] and Huang et al.[8] providing non-ATP competitive p56^{lck} and pp60$^{c\text{-}src}$ tyrosine kinase inhibitors.

In the current study, the crystal structure of the auto-inhibited human IRTK catalytic domain[9] was used to carry out qualitative molecular modeling studies (SYBYL^{TM}, 6.4, Tripos Inc., St. Louis) wherein a naphthalene ring was superimposed upon the IRTK Tyr 1,162. The IRTK region containing Tyr 1,162 folds back into the active site, with Tyr 1,162 positioned analogous to a phosphorylatable Tyr in a peptide substrate, thereby autoinhibiting the tyrosine kinase. This superimposition indicated that an amide carbonyl should be placed at the 2-position (Scheme 1) of the naphthalene ring to mimic the Tyr 1,162 carbonyl and a

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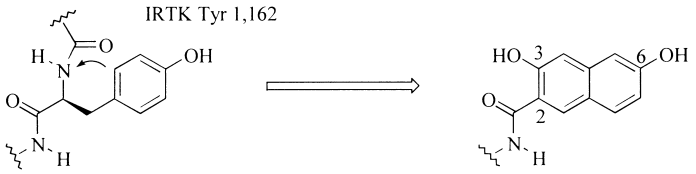
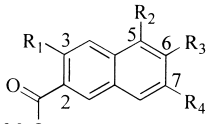


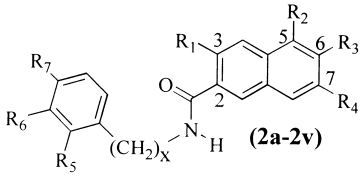
Scheme 1.

hydroxyl group should be positioned at the 6-position to mimic the Tyr 1,162 hydroxyl group. These modeling studies also indicated that a hydroxyl group at the 3-position could mimic the Tyr 1,162 NH.

In order to test these design concepts experimentally, the 2-position carbonyl group was appended as either a methyl ester or as a series of amides (Table 1). The hydroxy *N*-phenyl (X = 0) and *N*-benzyl (X = 1) amides were chosen based upon the increase in pp60$^{c\text{-}src}$ inhibitor potency observed with iminochromene analogues containing appended hydroxy *N*-phenyl amide side-chains.[8] Analogues wherein the 6-hydroxyl group was either deleted or moved were also prepared to determine if a drop in potency occurs as predicted from the modeling studies.

Table 1. pp60$^{c\text{-}src}$ Inhibitory activity of hydroxynaphthalene derivatives and select published inhibitors[a–c]





Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	X	% Inhibition at 100 μM (std. dev.)	IC_{50} (μM)
1a	OH	OH	H	H	N/A	N/A	N/A	N/A	5 (±2)	n.t.
1b	OH	H	OH	H	N/A	N/A	N/A	N/A	47 (±3)	n.t.
1c	OH	H	H	OH	N/A	N/A	N/A	N/A	19 (±6)	n.t.
1d	NH_2	H	H	H	N/A	N/A	N/A	N/A	Inactive	n.t.
2a	OH	OH	H	H	OH	H	H	0	12 (±4)	n.t.
2b	OH	OH	H	H	H	OH	H	0	51 (±1)	150
2c	OH	OH	H	H	H	H	OH	0	60 (±7)	n.t.
2d	OH	OH	H	H	OH	H	OH	0	14 (±2)	n.t.
2e	OH	H	OH	H	OH	H	H	0	39 (±5)	n.t.
2f	OH	H	OH	H	H	OH	H	0	89 (±1)	16
2g	OH	H	OH	H	H	H	OH	0	23 (±5)	n.t.
2h	OH	H	OH	H	OH	H	OH	0	56 (±1)	n.t.
2i	OH	OH	H	H	H	OMe	H	0	33 (±5)	n.t.
2j	OH	OH	H	H	H	H	OMe	0	35 (±8)	n.t.
2k	OH	OH	H	H	OMe	H	H	1	13 (±3)	n.t.
2l	OH	OH	H	H	H	H	OMe	1	14 (±2)	n.t.
2m	OH	H	OH	H	OMe	H	H	1	Inactive	n.t.
2n	OH	H	OH	H	H	H	OMe	1	4 (±7)	n.t.
2o	OH	OH	H	H	OH	H	H	1	41 (±2)	n.t.
2p	OH	OH	H	H	H	H	OH	1	49 (±4)	n.t.
2q	OH	H	OH	H	OH	H	H	1	42 (±2)	n.t.
2r	OH	H	OH	H	H	OH	H	1	55 (±3)	n.t.
2s	OH	H	OH	H	H	H	OH	1	42 (±3)	n.t.
2t	OH	H	H	OH	H	OH	H	0	68 (±5)	n.t.
2u	OH	H	H	OH	H	OH	H	1	40 (±3)	n.t.
2v	H	H	OH	H	H	OH	H	0	45 (±5)	n.t.
Iminochromene 9TA									30 (±15)	Lit[8]: 0.118
Piceatannol									41 (±2)	Lit[13]: 66 (lck)
ST-638									37 (±5)	Lit[14]: 18
Emodin[d]									22 (±3)	Lit[15]: 38
Tyrophostin A47									43 (±3)	

[a]The previously described assay procedure[11] was used with the following assay components, final concentrations and conditions: 50.0 mM MOPS, 4.02 mM $MgCl_2$, 6.00 mM K_3 citrate (used as a Mg^{2+} buffer to stabilize the free Mg^{2+} at 0.5 mM), 99.0 mM KCl, 10.0 mM 2-mercaptoethanol, 198 μM ATP, 19.8 μM ADP, 10 U full length human purified recombinant pp60$^{c\text{-}src}$ (Upstate Biotechnology Inc.), 2.00 mM RR-SRC, 4.0 % DMSO, pH 7.2, 37 °C. These overall assay conditions have been shown[12] to reproduce the intracellular conditions of pH, temp., free Mg^{2+} (0.5 mM), ionic strength, osmolality, ATP/ADP and reduction potential.
[b]All new compounds were characterized by proton NMR, EI or FAB(+) MS and are pure by TLC.
[c]N/A = Not applicable, n.t. = not tested.
[d]ATP-competitive.

The series of 2-carbonyl-3,5-dihydroxy naphthalene inhibitors (**1a**, **2a**–**2d**, **2i**–**2l**, **2o**–**2p**) and 2-carbonyl-3,7-dihydroxy naphthalene inhibitors (**1c**, **2t**–**2u**) were synthesized from commercially available (Aldrich) 3,5-dihydroxy-2-naphthoic acid and 3,7-dihydroxy-2-naphthoic acid, respectively. The methyl esters **1a** and **1c** were obtained by refluxing the respective acid starting materials for 48 h in methanol pre-saturated with HCl gas. The amides (**2a**–**2d**, **2i**–**2l**, **2o**–**2p**, **2t**–**2u**) were synthesized by coupling the respective carboxylic acid with commercially available (Aldrich or Lancaster) amines using one of two methods. The first method utilized the NBS/PPh_3 methodology as described by Froyen.[10] The second method utilized IIDQ (Aldrich) as the coupling reagent. The carboxylic acid was first reacted with 1.0 equiv IIDQ in anhydrous DMF at rt for 24 h. The respective amine (2.0 equiv) was then added neat and the reaction was heated to 80 °C for 2–6 h. After aqueous work up, purification was achieved by silica gel chromatography and precipitation from CH_2Cl_2/hexane, followed by preparative C-18 RP-HPLC (CH_3CN/H_2O), if necessary. The benzyl amines were commercially available only as their corresponding hydroxyl protected methyl ethers. Consequently, after amide formation, the hydroxyl groups were deprotected by treatment with 6 eq. BBr_3 in DCM for 1 min at −78 °C followed by 1 h at rt.

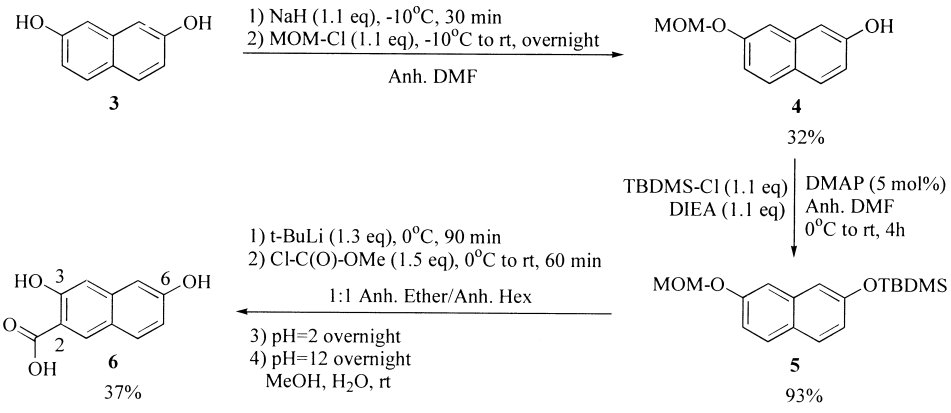
The series of 2-carbonyl, 3,6-dihydroxy naphthalene inhibitors (**1b**, **2e**–**2h**, **2m**–**2n**, **2q**–**2s**) was synthesized from 3,6-dihydroxy-2-naphthoic acid **6** using the methods described above. The synthesis of intermediate **6** that was developed is shown in Scheme 2 beginning with commercially available 2,7-dihydroxynaphthalene **3** (Aldrich).

Compound **1d** was synthesized from 3-amino-2-naphthoic acid (Aldrich) by reaction with TMS-diazomethane in DCM at room temperature. Compound **2v** was synthesized from 6-hydroxy-2-naphthoic acid (Aldrich) using the amidation method described by Froyen.[10]

Kinase assay conditions have been shown to influence the measured inhibitory activity.[1] Consequently, in order to accurately determine the relative potency of our newly designed class of $pp60^{c\text{-}src}$ inhibitors, we also tested the inhibitory activity of four previously published, non-ATP competitive PTK inhibitors. Piceatannol, ST-638, and Tyrphostin A47 were chosen because they are commercially available (Sigma or Calbiochem), and are representative of the spectrum of known non-ATP competitive PTK inhibitors. Emodin (Calbiochem) is ATP-competitive when analyzed with the tyrosine kinase $p56^{lck}$. Prior to this communication, iminochromene 9TA was the most potent non-ATP competitive $pp60^{c\text{-}src}$ inhibitor reported.[8] Since iminochromene 9TA was not commercially available, it was synthesized using a novel route,[16] and included in this study.

The inhibitory activities shown in Table 1 for compounds **1a**–**d** and **2a**–**2v** were determined using purified, full length, human recombinant $pp60^{c\text{-}src}$. Due to the number of compounds tested, and the associated cost, their rank order potencies were first determined at a constant inhibitor concentration (100 μM). As predicted by the modeling studies, based upon analogy to the IRTK Tyr 1,162 hydroxyl group, a preference for positioning the naphthalene hydroxyl group on carbon 6 versus 5 or 7 was observed in both the ester (**1b**, 47% versus **1a**, 5% & **1c**, 19%) and amide (e.g. **2f**, 89% versus **2b**, 51% and **2t**, 68%) series. The prediction that attaching a hydroxyl group at naphthalene carbon 3 (mimicking the Tyr 1,162 NH) would improve potency was also confirmed (**2f**, 89% versus **2v**, 45%). Finally, the prediction that extending the inhibitor as an amide at the 2 position (mimicking the peptide bond) could further improve potency was confirmed as well (e.g. **2f**, 89% versus **1b**, 47%).

The data provided in Table 1 demonstrate that moving the hydroxyl group from the optimal 6 position to the adjacent naphthalene carbon 5 results in a different structure activity profile with regard to the optimal concurrent positioning of the hydroxyl group(s) in the amide side chain (e.g. **2f**/**2g** versus **2b**/**2c**). Also of note is the replacement of the amide side chain hydroxyl group with a corresponding methoxy group in compounds **2i**–**2n**. In the case of the *N*-phenyl amides (**2i**–**2j**), their



Scheme 2.

activity, relative to the corresponding hydroxy amides (**2b**–**2c**), was not reduced as significantly as in the case of the *N*-benzyl amides (**2k**–**2n** versus **2o**–**2q**, **2s**). This suggests that in the benzyl derivatives, the amide side chain hydroxyl groups either interact with the enzyme as hydrogen bond donors, or the methoxy groups are too large to fit in the binding site.

A more quantitative analysis of the selectivity for positioning a hydroxyl group on carbon 6 versus 5 is provided by comparing the IC_{50}'s of **2f** (16 μM) versus **2b** (150 μM), respectively. These results also confirm that a drop in % inhibition from ca. 90 to 50% represents an order of magnitude difference in potency, as expected. Similarly, a drop in % inhibition from ca. 50 to 10% would represent another order of magnitude difference in potency.

A direct comparison of the most potent inhibitor from this series, compound **2f**, with the five previously reported PTK inhibitors shown in Table 1 demonstrates that, under these assay conditions, **2f** is more potent by one to two orders of magnitude. Interestingly, iminochromene 9TA was previously reported[8] to have an IC_{50} of 118 nM against $pp60^{c\text{-}src}$, and was the most potent known non-ATP competitive $pp60^{c\text{-}src}$ inhibitor, but under the current assay conditions only a 30% inhibition at 100 μM was observed. These results re-emphasize[1] the importance of comparing protein kinase inhibitors under identical assay conditions.

A goal of these studies was to obtain non-peptide $pp60^{c\text{-}src}$ inhibitors which do not compete with ATP. Consequently the % inhibition of $pp60^{c\text{-}src}$ by **2f** and **2b** at constant inhibitor concentrations was monitored as a function of increasing [ATP] up to a cellular mimetic 1 mM level. Since the [ATP] had little effect on the % inhibition, both **2f** and **2b** are non-competitive inhibitors with respect to ATP under these assay conditions.[17] The high cost of many kinases has stimulated other researchers to monitor inhibitor potency as a function of increasing [ATP] for the same purpose.[6,7,18–21]

In summary, structure-based design has produced a series of hydroxynaphthalene $pp60^{c\text{-}src}$ non-peptide inhibitors which do not compete with ATP. Results with compounds from this series in cell-based assays, as well as detailed kinetic studies under various assay conditions, will be reported in due course. An extension of these design concepts from the naphthalene scaffold to an indole scaffold is reported in the following paper.

Acknowledgements

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16. 3-Aminophenol was converted to the corresponding TBDMS ether (1.1 equiv TBDMS-Cl, 1.1 equiv DIEA, 5 mol% DMAP, DMF, 24 h, rt, 71%). The resulting aniline was coupled using 2.0 equiv of cyanoacetic acid (1.1 equiv EDCI, 1.1 equiv TEA, DMF, 18 h, 75 °C, 70%). Condensation of the resulting amide with 1.2 equiv. of 2,3-dihydroxybenzaldehyde (cat. piperidine, abs. EtOH, 2 h, 60 °C) followed by deprotection (1.1 equiv. TBAF, THF, 15 m, 43% overall) gave iminochromene 9TA with satisfactory elemental, FAB(+)MS and ^{1}H NMR analysis after purification by flash chromatography (10:1, DCM:MeOH).
17. The % inhibition was measured using ATP concentrations of 200, 500 and 1000 μM while holding the inhibitor concentration constant. If the inhibitor is directly competing with ATP, then this 5-fold overall increase in [ATP] is equivalent to decreasing the inhibitor concentration 5-fold in terms of the effect on % inhibition. Consequently the % inhibition should decrease to the value observed in the IC_{50} dose–response curve (obtained with 200 μM ATP) for 1/5 of the set inhibitor concentration used in this experiment if direct competition with ATP is occurring. For inhibitor **2f** (set at 25 μM) a 62% (±5), 54% (±3) and 50% (±1) inhibition at 200, 500 and 1000 μM ATP, respectively, was obtained whereas the level of inhibition should have dropped to ca. 20% at 1000 μM ATP if direct competition with ATP were occurring. Similarly, for inhibitor **2b** (set at 300 μM) an 84% (±1), 81% (±1) and 77% (±2) inhibition at 200, 500 and 1000 μM ATP, respectively, was obtained.
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