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Inhibition of Src Kinase Activity by 4-Anilino-5,10-dihydro-pyrimido[4,5-*b*]quinolines

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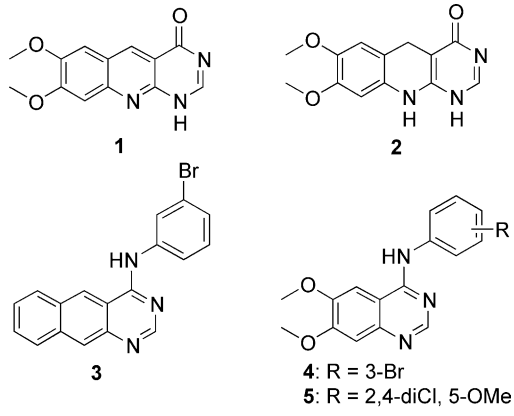
Abstract—4-(2,4-Dichloro-5-methoxy)anilino-5,10-dihydropyrimido[4,5-*b*]quinolines are potent inhibitors of Src kinase and Src cellular activity while having no effect on Fyn cellular activity. The corresponding 4-(2,4-dichloro-5-methoxy)anilino-pyrimido[4,5-*b*]quinolines are much less effective Src inhibitors.

Protein tyrosine kinases (TKs) catalyze the transfer of a phosphate group from ATP to a specific tyrosine residue on a protein. Since aberrant TK activity is associated with a variety of disease states, there is great interest in investigating low molecular weight inhibitors of TK activity as potential agents for the treatment of cancer and other conditions.[1] Several years ago, a group at Pfizer reported that while the pyrimido[4,5-*b*]quinolin-4(1*H*)-one, **1**, was a moderate Src kinase inhibitor, the corresponding 5,10-dihydro analogue, **2**, was 60-fold more potent (IC_{50} = 32 and 0.5 μM respectively).[2] Interestingly when studying a series of EGFr inhibitors, Parke-Davis (now Pfizer) found that the tricyclic analogue **3** was more potent than the bicyclic analogue **4**.[3] We had earlier shown that changing the 3-bromo aniline substituent of **4** to 2,4-dichloro-5-methoxy changed the kinase selectivity, with **5** now being a Src inhibitor.[4] We therefore envisioned that addition of the 2,4-dichloro-5-methoxyaniline group present in **5** to the C-4 position of **1** and **2** might increase the Src inhibitory activity of these compounds. Based on what was seen with **3** and **4**, these tricyclic analogues of **5** may also be more potent than the parent bicyclic derivative.

The preparation of the initial target compounds is shown in Scheme 1. Treatment of 2-amino-6,7-dimethoxy-3-quinolinecarbonitrile[5] with dimethyl-formamide dimethyl acetal gave the corresponding amidine. Subsequent addition of 2,4-dichloro-5-methoxyaniline in acetic acid provided the desired tricyclic derivative **6**.[6] Treatment of **2** with phosphorous oxychloride provided the intermediate 4-chloro derivative which was reacted with 2,4-dichloro-5-methoxyaniline in 2-ethoxyethanol in the presence of pyridine-HCl to provide the reduced analogue **7**.



As shown in Table 1, **7** is more than 3000-fold more potent in inhibiting Src kinase activity than **6**, with **7** having an IC_{50} of 3.3 nM in this assay.[7] This dramatic difference was also observed in cells, with **7** strongly inhibiting the proliferation of Src transformed fibroblasts (IC_{50} = 190 nM). The disparities in the activities seen with **6** and **7** are much more pronounced than those observed by Pfizer with the 4-one derivatives **1** and **2**.

Addition of a water solubilizing group to **7** would be expected to improve the cell activity of this compound. To this end, as shown in Scheme 2, a 2-methoxyethoxy

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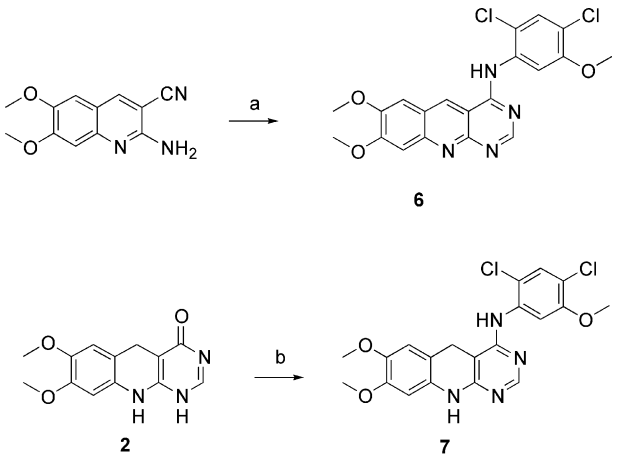
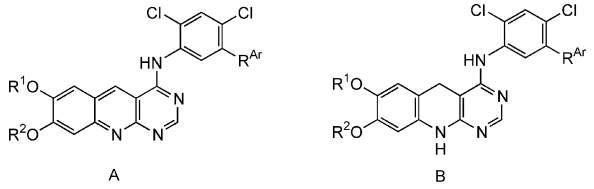


Scheme 1. (a) (1) DMF-DMA; (2) 2,4-diCl-5-OMe aniline, AcOH; (b) (1) $POCl_3$; (2) 2,4-diCl-5-OMe aniline, pyridine-HCl, 2-ethoxyethanol.

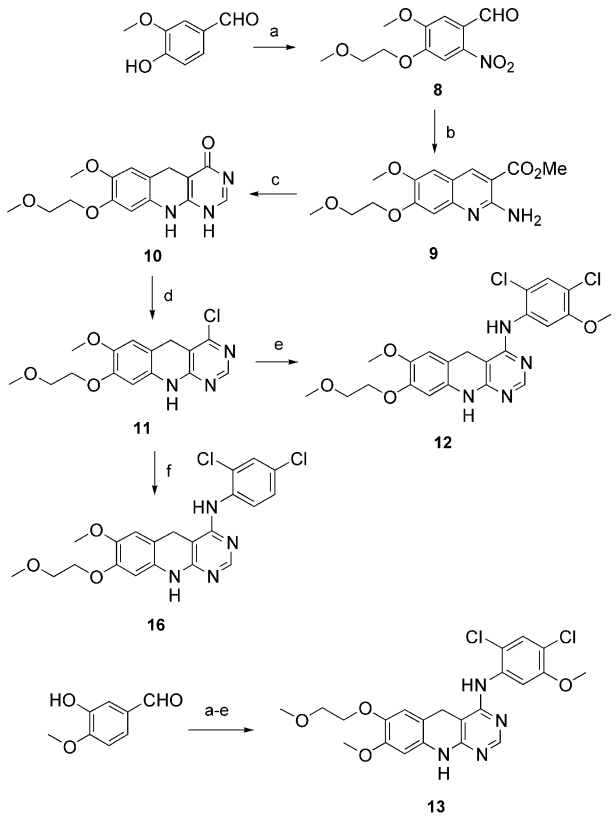
Table 1.



Compd	Ring	R^1	R^2	R^{Ar}	Src enzyme[7] (IC_{50} nM)	Src cells[7] (IC_{50} nM)
6	A	Me	Me	OMe	>10,000	>10,000
7	B	Me	Me	OMe	3.3	190
12	B	Me	CH_2CH_2–OCH_3	OMe	2.3	28
13	B	CH_2CH_2–OCH_3	Me	OMe	9.8	140
15	A	Me	CH_2CH_2–OCH_3	OMe	35% at 5 μM	>10,000
16	B	Me	CH_2CH_2–OCH_3	H	16	120
18					1.2	100

group was added to C-7 and C-8 of **7** to provide **12** and **13**, respectively. The 2-methoxyethoxy group is the solubilizing group present on Tarceva, a 4-anilinoquinazoline EGFr inhibitor currently undergoing clinical trials.[8]

Alkylation of vanillin with 2-chloroethyl methyl ether followed by nitration provided **8**. Condensation of **8** with methyl cyanoacetate and subsequent reduction with iron provided the bicyclic derivative **9**. Cyclization of **9** with formamide at high temperatures gave the desired tricyclic core **10**. Chlorination of **10** with phosphorous oxychloride provided **11**. Attempts to add 2,4-dichloro-5-methoxyaniline under the standard conditions of pyridine hydrochloride failed to provide the desired product. It should be noted that although these were the conditions used to prepare **7**, the yield in this reaction was only 22%. Fortunately, implementation of the palladium catalyzed coupling conditions reported by Buchwald gave **12** in 64% yield.[9]

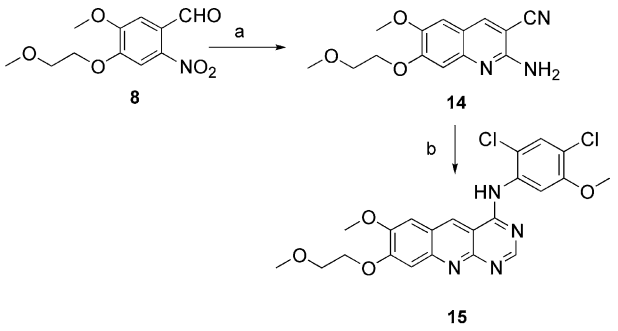


Scheme 2. (a) (1) 2-Chloroethyl methyl ether, K_2CO_3, DMF; (2) fuming HNO_3, dichloroethane; (b) (1) methyl cyanoacetate, piperidine, MeOH; (2) Fe, AcOH; (c) $HCONH_2$; (d) $POCl_3$; (e) 2,4-diCl-5-OMe aniline, tris(dibenzylidene)acetate, 2-dicyclohexylphosphino-2′-(*N*,*N*-dimethylamino)biphenyl, K_3PO_4, DME; (f) 2,4-diCl aniline, tris(dibenzylidene)acetate, 2-dicyclohexylphosphino-2′-(*N*,*N*-dimethylamino)-biphenyl, K_3PO_4, DME.

The isomer of **12**, namely **13**, where the solubilizing group is at C-7 was prepared in an analogous fashion from 3-hydroxy-4-methoxybenzaldehyde. As for the preparation of **12**, the final product, **13**, was obtained via a palladium catalyzed coupling reaction.

As shown in Table 1, **12**, the analogue with the solubilizing group at C-8, had about a 4-fold increase in both Src enzymatic and cell activity compared to **13**. For comparision purposes, the analogue of **6** with a 2-methoxyethoxy group at C-8 was prepared as shown in Scheme 3. Intermediate **8** was reacted with malononitrile in the presence of β-alanine. Reduction with iron in acetic acid provided **14**. Formation of the amidine derivative with dimethylformamide dimethyl acetal followed by addition of the aniline gave **15**, the aromatic analogue of **12**. As shown in Table 1, **15** was of greatly reduced activity compared to **12**.

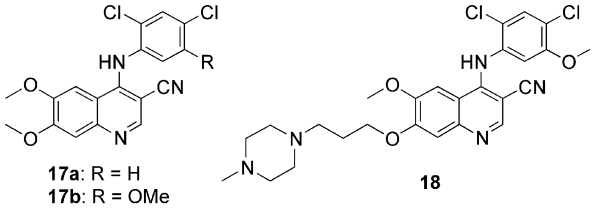
We earlier reported that while the optimal substituent on the C-4 aniline was 2,4-dichloro-5-methoxy, the 2,4-dichloroaniline derivative also provided Src inhibition.[4] Analogue **16** was prepared by addition of 2,4-dichloroaniline to **11** under the palladium catalyzed conditions used to prepare **12**. When tested in the Src enzyme and cell assays **16** was about 4-fold less potent than **12**. This corresponds to what was observed in a



Scheme 3. (a) (1) Malononitrile, β-alanine, MeOH; (2) Fe, AcOH; (b) (1) DMF–DMA; (2) 2,4-diCl-5-OMe aniline, AcOH.

series of 3-quinolinecarbonitrile Src inhibitors where **17b** was about 7-fold more potent than **17a** when tested in the enzymatic assay (IC_{50}s of 4.3 and 30 nM, respectively).[4]

Optimization of **17b** for cell activity and plasma levels after oral dosing led to **18**, which had an IC_{50} in the Src cell assay of 100 nM (see Table 1).[4] This compound was 4-fold less potent in inhibiting the proliferation of Fyn-dependent cells (IC_{50} of 410 nM). Fyn is a Src family kinase (SFK) and shares a high degree of structural homology with Src. Of all the additional 3-quinolinecarbonitrile Src inhibitors we reported previously, we did not observe greater than 10-fold selectivity for Src over Fyn.[10–12] It was therefore very surprising to find that **12** did not inhibit the proliferation of Fyn-dependent cells ($IC_{50} > 10$ μM). Furthermore, no inhibition of Fyn-dependent cell growth was observed with **7**, **13**, **15** or **16**.



To further investigate this unpredicted finding, **12** was tested for its ability to inhibit the phosphorylation of cortactin, a natural substrate for SFKs. As shown in Figure 1, while **12** blocked the phosphoryation of tyrosine 421 of cortactin in a dose-dependent fashion in the Src cells, this compound had no effect on cortactin phosphoryation in the Fyn cells.[13]

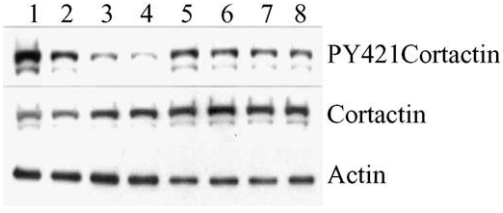


Figure 1. Selective inhibition of cortactin phosphorylation on tyrosine 421 by **12**. Immunoblot of extracts from Src and Fyn transformed Rat2 fibroblasts exposed to **12** for 5 h. Lysates from Src-transformed (lanes 1–4), and Fyn-transformed (lanes 5–8) Rat2 fibroblast cells. Lanes 1 and 5, no treatment; lanes 2 and 6, 0.1 μM **12**; lanes 3 and 7, 0.5 μM **12**; lanes 4 and 8, 1.0 μM **12**.

While the Src enzymatic activity of **12** is comparable to that of **18**, **12** is at least 3 times more potent in the Src cell assay. However, in spite of the cellular potency and Src selectivity of **12**, this compound was not active when tested in an in vivo nude mouse xenograft model employing the Src-transformed fibroblasts.[4] In attempts to measure plasma levels in nude mice after administration of **12**, no parent compound could be detected. It was observed that while **12** was stable in the solid state, solutions of **12** rather quickly oxidized to give **15**. The investigation of more stable analogues of **12** is underway. We also hope to determine the reason for the increased Src cell activity observed with these analogues and for their inactivity against Fyn.

Acknowledgements

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transformed Rat2 fibroblasts were prepared as previously described.[4] Compound **12** was freshly dissolved in DMSO immediately before addition to medium. Cells were exposed to **12** for 5 h. Lysates were analyzed on Novex 4–12% gradient gels and transferred to PVDF membrane. Antibodies were obtained from Upstate Biotechnology (anti-Cortactin), BioSource (anti-pY421 Cortactin), and Chemicon International (anti-Actin).