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3,5,6-Trisubstituted Naphthostyrils as CDK2 Inhibitors

Jin-Jun Liu,[a,*] Apostolos Dermatakis,[a] Christine Lukacs,[a] Fred Konzelmann,[a] Yi Chen,[a] Ursula Kammlott,[a] Wanda Depinto,[b] Hong Yang,[b] Xuefeng Yin,[b] Yingsi Chen,[b] Andy Schutt,[b] Mary Ellen Simcox[b] and Kin-Chun Luk[a]

[a]*Department of Discovery Chemistry, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110, USA*
[b]*Department of Oncology, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110, USA*

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Abstract—A novel class of 3,5,6-trisubstituted naphthostyril analogues was designed and synthesized to study the structure–activity relationship for inhibition of cyclin-dependent kinase 2 (CDK2). These compounds, particularly molecules with side-chain modifications providing additional hydrogen bonding capability, were demonstrated to be potent CDK2 inhibitors with cellular activities consistent with CDK2 inhibition. These molecules inhibited tumor cell proliferation and G1-S and G2-M cell-cycle progression in vitro. The X-ray crystal structure of a 2-aminoethyleneamine derivative bound to CDK2, refined to 2.5A resolution, is presented.

Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases that govern the initiation, progression and completion of the cell cycle. Activity of the CDKs allows orderly transition between phases of the cell cycle.[1] CDK activity is controlled by association with regulatory subunits (cyclins) and CDK inhibitor proteins,[2] by their phosphorylation state and by ubiquitin-mediated proteolysis.[3] As drivers of the cell cycle and cell proliferation, CDKs represent attractive therapeutic targets for treatment of cancer.

Misregulation of CDKs occurs with high frequency in the major solid tumor types including breast, colon, prostate, non-small cell lung and ovarian carcinomas.[4] Small molecule inhibitors of CDKs that block cell cycle progression could potentially lead to effective antitumor agents and could fill a large unmet medical need.[5] A number of small molecule inhibitors of CDK2 have been reported.[6] We recently described the chemistry of a new class of compounds, 3,5,6-trisubstituted naphthostyrils, as CDK2 inhibitors.[7] Herein, we report the design, synthesis, and initial biological evaluation of this novel series.

In early efforts to identify small molecule inhibitors of CDK2, we[8] and others[9,10] found that oxindole derivatives **1** and **2** showed potent CDK2 as well as tyrosine kinase inhibitory activities. The chemical stability of these oxindoles, however, could be a potential issue for drug development. While the *Z* form is stabilized by an intramolecular hydrogen bond, equilibrium between the *Z* and *E* isomers in polar solvents, such as methanol and dimethyl sulfoxide, or in the presence of light has been observed[10] (Fig. 1).

Since the *Z* configuration is the preferred form for the inhibitory activity of these compounds against CDK2,[10] it would be ideal to fix this *Z* configuration. To this end,

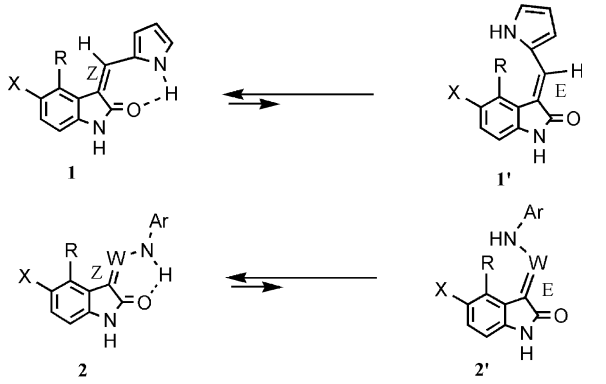

Figure 1.

*Corresponding author. Tel.: +1-973-235-2851; fax: +1-973-235-7122; e-mail: jin-jun.liu@roche.com

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we have designed conformationally constrained 3-pyrrolyl naphthostyrils **4** in which a two-carbon bridge has been introduced between the vinyl carbon and the aromatic carbon at the C-4 position of the original oxindole **3** to form an extra ring (Fig. 2).

The first analogue made in this series was compound **4a,** 5-fluoro-3-pyrrolylnaphthostyril. This compound, as expected, was very stable and maintained the potency of the lead compound (oxindole **3**) in the CDK2 enzyme assay as shown in Figure 2. In addition, the chemistry we developed for the preparation of compound **4** allows the rapid synthesis of analogues with diverse substituents at the 5-position of the naphthostyril ring for exploration of SAR.

Early modeling studies suggested that such analogues could have increased potency. It was also thought that incorporating a side chain with one or more heteroatoms, such as O, N and S, onto the ring system would further result in analogues with improved solubility and ADME properties. We therefore designed and synthesized structures **6**, **7**, **8**, **9** and **10** starting with β-iodovinyl ketone **5** as the common intermediate (Scheme 1).

The first set of compounds we made for probing the SAR at C_5, represented by the ether **6**, were prepared by the reaction of β-iodovinyl ketone **5** with the appropriate sodium alkoxide (ROH, NaH, neat, heating).[7a] The biological activity data from this set of inhibitors revealed that addition of an ether side chain enhanced the potency dramatically (Table 1, **6a**–**6e**). For example, by simply introducing a methoxy group at the 5-position, compound **6a** was 10-fold more potent against CDK2 compared to compound **4a**. Increasing the bulk of the alkyl group tended to decrease the potency in vitro (**6b** vs **6a**). However, incorporating a functional group such as OH or NH_2 at the end of the side chain increased potency (**6c**–**e** vs **6b**), suggesting these functional groups may pick up extra hydrogen bonds within the CDK2 binding pocket. This was further evidenced by the poor activity of **6f** in which the OH group was blocked.

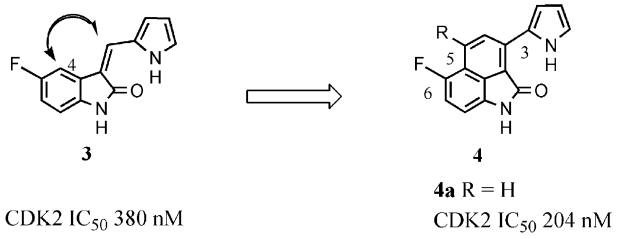
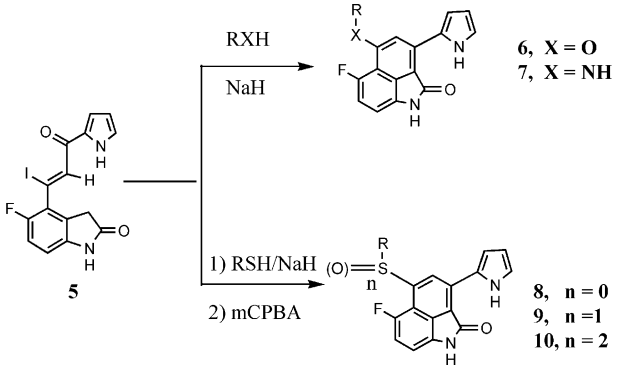


Figure 2.

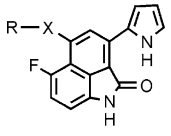


Scheme 1.

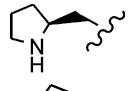
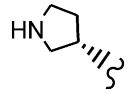
We next incorporated alkylamino substituents at the 5-position of the naphthostyrils by using the same type of chemistry (RNH_2, NaH, neat, heating). Consistent with the SAR established in the ether series, compounds possessing an alkyl amine substituent at the 5-position of the naphthostyrils were more active than the parent compound **4a** (Table 1, **7a**–**7d**, **7f**). Likewise, certain hydrogen bond donating functional groups at the end of the side chain increased potency as in the ether case (**7a**–**7d**). Partially blocking the hydrogen bond donating capability of these groups by acylation dramatically reduced their activity (**7f**–**g** vs **7b**). Alkylation of the amino group also decreased the potency (**7e** vs **7b**). Compound **7b** was one of the most potent derivatives in the series. Increasing the length of the side chain (**7c** vs **7b**) or introducing a gem dimethyl group next to the end amino group (**7d** vs **7b**) led to a 5- to 7-fold reduction in potency.

A similar approach was used in the synthesis of the third set of analogues, the thio-ethers **8,** from the β-iodovinyl ketone **5** and RSH (RSH, NaH, neat, heating). The

Table 1. In vitro potency of naphthostyrils as CDK2 inhibitors



6, X = O
7, X = NH
8, X = S
9, X = S(=O)
10, X = SO_2

Compd	R	IC_{50} (nM)
6a	Me	18±6
6b	Et	77[a]
6c	$HOCH_2CH_2$	62±10
6d	$NH_2CH_2CH_2$	12±7
6e	$HOCH_2CH_2CH_2$	18±3
6f	$AcOCH_2CH_2CH_2$	>1000[a]
7a	$HOCH_2CH_2$	54±19
7b	$NH_2CH_2CH_2$	12±10
7c	$NH_2CH_2CH_2CH_2$	48±58
7d	$NH_2C(CH_3)_2CH_2$	65±41
7e	$Et_2NCH_2CH_2$	294±256
7f	$AcNHCH_2CH_2$	127±94
7g	$BocNHCH_2CH_2$	5500[a]
8a	$HOCH_2CH_2$	14[a]
8b	$NH_2CH_2CH_2$	3±2
8c		18[a]
8d		5[a]
9b	$NH_2CH_2CH_2$	28±8
10a	$HOCH_2CH_2$	15[a]
10b	$NH_2CH_2CH_2$	25±3

[a]IC_{50} values were estimated with at least two replicates at each concentration.

corresponding sulfoxides, **9**, or sulfones, **10**, were obtained by oxidation of compounds **8**. For example, treatment of compound **5** with $HOCH_2CH_2SH$ in the presence of NaH at 120 °C, gave the 5-(2-hydroxyethylthio)-naphthostyril, **8a**, which was oxidized by mCPBA to yield the corresponding sulfoxide **9a** or sulfone **10a**. In general, the thioether, sulfoxide and sulfone naphthostyril analogues had comparable activity with their ether or alkylamino counterparts (Table 1).

By comparing the activities of compounds **6**, **7** and **8**, it was observed that there was no significant difference among the three series, suggesting that the heteroatom linker made no direct contribution to the CDK2 inhibitory activities (**6c** vs **7a** vs **8a**, and **6d** vs **7b** vs **8b**).

For comparison, analogues of compound **4** containing alkyl substituents at the 5-position of the naphthostyril ring were also investigated. The synthesis of this set of analogues required the formation of a C-C bond, which was achieved by treatment of the common intermediate **5** with organometallic reagents such as organocoppers or alkylzinc-copper.[7b] The CDK2 inhibitory activities of this set of compounds are shown in Table 2. The alkyl substituents were also well tolerated in the CDK2 enzyme assay. Again, functional groups at the end of the side chain dramatically increased the activities as shown in previous cases (**4e** and **4f**).

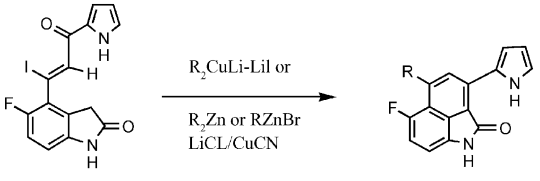
In order to confirm the binding mode for the tri-substituted naphthostyrils and to understand the role of the side chain, we determined the X-ray crystal structure of CDK2 in complex with compound **7b** to 2.5 Å resolution. As anticipated, the hydrogen bond donor-acceptor pair binding to the hinge region of the ATP binding site of CDK2 was comprised of the naphthostyril moiety. The oxindole carbonyl oxygen accepted a hydrogen bond from the backbone N of Leu 83 and the oxindole nitrogen donated a hydrogen bond to the backbone carbonyl of Glu 81. The pyrrole nitrogen provides a third hinge region hydrogen bond to the backbone carbonyl of Leu 83. This nitrogen was also involved in an intramolecular hydrogen bond with the oxindole carbonyl, enforcing the co-planarity of the pyrrole ring with the naphthostyril core. Figure 3 showed the X-ray crystallographic structure of CDK2 in complex with compound **7b**, superimposed with a published oxindole derivative (**2**, X = Br, R = H, W = N, Ar = $-NH_2SO_2Ph-$).[11]

Although it was oriented out of the binding cleft, the alkylamino side chain of **7b** also provided an additional hydrogen bond between its terminal amine and Asn 132. This extra hydrogen bond explained the increased potency of such compounds. Finally, as was seen with most kinase inhibitors, the planar core made a number of Van der Waals contacts to the hydrophobic binding cleft of the protein. For example, residues lining the pocket Ile 10, Val 18, Ala 31, Val 64, Phe 80, and Leu 134 were all less than 4 Å from the inhibitor.[12]

Additional evidence that this new class of compounds inhibits CDK2 enzyme activity in cells was obtained using proliferation assays, cell-cycle analysis and a mechanistic assay that measures progression of cells from G1 to S phase.[6b] The compounds inhibited the proliferation of tumor cells, blocked progression of G0 synchronized cells into S phase and arrested asynchronous cells in G1 + G2 of the cell cycle. This is consistent with the mechanism of action of a CDK2 inhibitor (Table 3).

In conclusion, by introducing an aromatic ring into the oxindole derivative **3**, we have discovered a new series of potent CDK2 inhibitors, 3,5,6-trisubstituted naphthostyrils. In general, a side chain at the 5-position of naphthostyrils increased CDK2 inhibitory activity. Compounds with a functional group at the end of the side chain show the most potent activity against CDK2 and are potentially useful as anticancer agents in humans.

Table 2. In vitro potency of naphthostyrils as CDK2 inhibitors



5 **4**

Compd **4**	R	IC_{50} (nM)
a	H	204 ± 3
b	Me	376[a]
c	Et	72 ± 1
d	*i*-Bu	124 ± 168
e	$NCCH_2CH_2$	5[a]
f	$NH_2CH_2CH_2CH_2$	7[a]

[a]IC_{50} values were estimated with at least two replicates at each concentration.

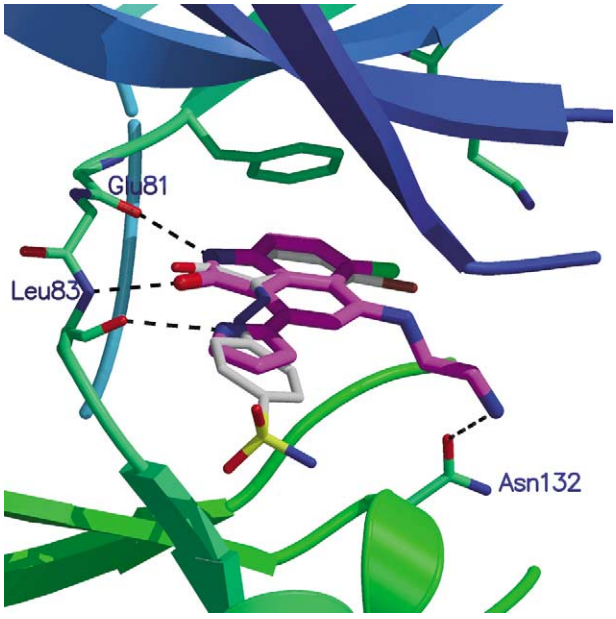


Figure 3. Superimposition of **7b** (magenta) with **2**[11] (gray) shows the binding mode to CDK2.

Table 3. Cellular assay data (IC_{50} μM)[a]

Compd	MTT[b]		BrdU[e]	Cell cycle[f]
	RKO[c]	MDA MB-435[d]		
4a	5.95	2.28	>30.0	
6a	0.75	0.89	7.1	G1 + G2
6c	0.50	0.58	10.87	
6d	0.11	0.38	0.91	G1 + G2
7a	0.74	0.62	2.39	
7b	0.08	0.16	1.49	G1 + G2
7c	0.12	0.07	2.04	
7d	0.11	0.16	1.67	
8a	1.48	1.73	2.74	
8b	0.10	0.31	0.81	G1 + G2
8c	0.06	0.21		
8d	0.07	0.12		
10a	1.20	3.20		
9b	0.42	1.21	1.87	
10b	0.43	1.08		

[a]IC_{50} values were estimated with at least two replicates at each concentration.
[b]Tumor-cell proliferation.
[c]Human colon cancer cell line.
[d]Human breast carcinoma cell line.
[e]G1-S cell-cycle progression assay.
[f]Flow cytometry.

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12. Crystals of the CDK2:**7b** complex were grown at 4 °C by the vapor diffusion method. CDK2(1–298) at 12 mg/mL was mixed with and equilibrated against 10% PEG 3350, 0.2 M ammonium phosphate, 0.1 M Bicine pH 9.0. 2% β-mercaptoethanol was added to the reservoir after mixing of the drop. Cryoprotectant was the same as the reservoir with 20% PEG 3350 and the addition of 15% ethylene glycol. X-ray data was collected at beamline X8C at Brookhaven National Laboratories. Data was processed to 2.5 Å with the HKL package (Otwinowski, Z., Minor, W. *Methods Enzymol.* **1997**, *276*, 307) to an R-sym of 0.071. The structure was determined by molecular replacement using AmoRe (Navaza, J. *Acta Crystallogr.* **1994**, *A50*, 157) and was refined with CNX (CNX v.99.0-BETA, Molecular Simulations Inc.) to an R-factor/Rfree of 0.208/0.279. Coordinates have been deposited to the PDB under accession code 1p2a.