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A new series of potent oxindole inhibitors of CDK2

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Abstract—A novel series of oxindole-type inhibitors of CDK2 that have heteroatom substituted alkynyl moieties at their C-4 position is described. These novel 4-alkynyl-substituted inhibitors have superior potency relative to their parent compound in free enzyme and in cell based assays. The crystal structure of CDK2 in complex with one of these analogues was determined and gives insight to their increased potency. The biochemical evaluation of a representative derivative is also described.

Cyclin-dependent kinase 2 (CDK2) is a key cell cycle regulator. Upon complexation with its activating proteins, cyclin E or cyclin A, CDK2 modulates the activity of many cellular substrates via phosphorylation on Ser and/or Thr residues.[1] In complex with cyclin E, CDK2 plays a paramount role during the G1/S transition of the cell cycle while in complex with cyclin A it facilitates the progression of the S phase of the cell cycle. Recent evidence also suggests that CDK2 may have a crucial role in the G2 phase of the cell cycle.[2] The importance of CDK2 for cell cycle progression has led to an active pursuit of small molecule inhibitors of this enzyme as a possible treatment against cancer and other hyperproliferative disorders.[3,4]

We recently disclosed a potent series of oxindole inhibitors of CDK2 that is based on the screening hit **1a**.[5] Herein we describe a new series of oxindole type inhibitors of CDK2 based on the lead **1b** (Fig. 1, Table 1).

Our work toward the potency optimization of **1b** started by reviewing the CDK2-ATP co-crystal structure in conjunction with several published crystal structures of CDK2 in complex with oxindole-type inhibitors.[6] This review lead us to assume that lead **1b**, like other oxindoles, must bind in the CDK2 ATP pocket along the residues Glu81–Leu83 in a donor acceptor donor motif. In that orientation, an overlay of **1b** with ATP in the CDK2 active site led us to the conclusion that the potency of this lead could be improved by the introduction of an appropriate heteroatom substituted moiety at C-4.

By means of this strategy and via the incorporation of cyclic saturated heteroatom substituted moieties at C-4, we had previously effected the potency optimization of screening hit **1a**.[5] In the case of the screening hit **1b** we decided to follow a slightly different approach for its potency optimization. Instead of pursuing the introduction of cyclic appendages at its C-4 position we opted to explore the use of heteroatom substituted alkynyl moieties. Through modeling we came to the realization that a heteroatom substituted propargyl and/or homopropargyl appendage at C-4 could be as beneficial for potency as were the cyclic moieties used during the optimization of lead **1a**.

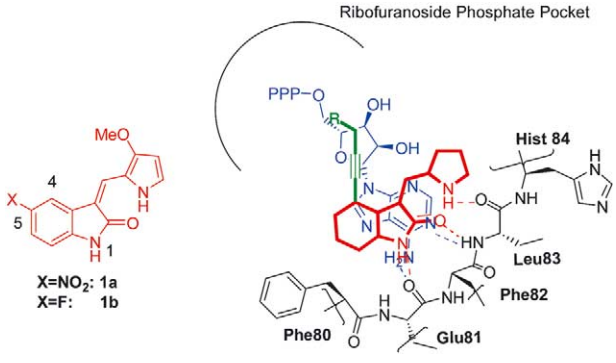

Figure 1. Screening hits **1a**,**b**. Overlay of the core of lead **1b** with ATP in the CDK2 pocket and visual representation of its optimization strategy. Interactions between CDK2 and the ribofuranoside phosphate moiety of ATP have been omitted for clarity.

Keywords: CDK2; CDK Inhibitors; Oxindole.
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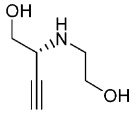
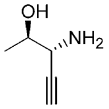
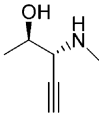
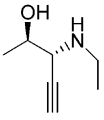
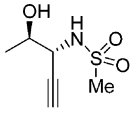
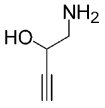
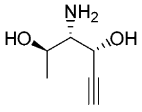
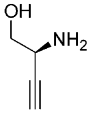
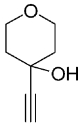
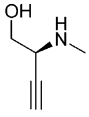
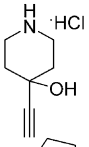
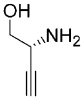
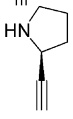
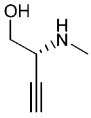
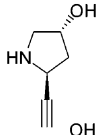
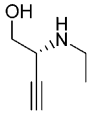
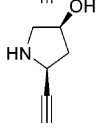
The synthesis of 4-alkynyl derivatives of **1b** was carried out as described in Scheme 1.[7] Via that route we prepared the 5-fluoro-4-alkynyl analogues **7a**–**t** shown in Table 1.

Assay results of 4-alkynyl derivatives **7** against the CDK2/cyclin E holoenzyme and against the cancer cell lines SW480 and MDA MB 435 revealed that these analogues were much superior to the lead compound **1b**.[8] In the CDK2 enzyme assay the great majority of alkynyl oxindoles **7** had an IC_{50} in the single digit nanomolar range. The cell based assays showed that these analogues were potent inhibitors of cellular proliferation, consistently better than **1b** by one or two

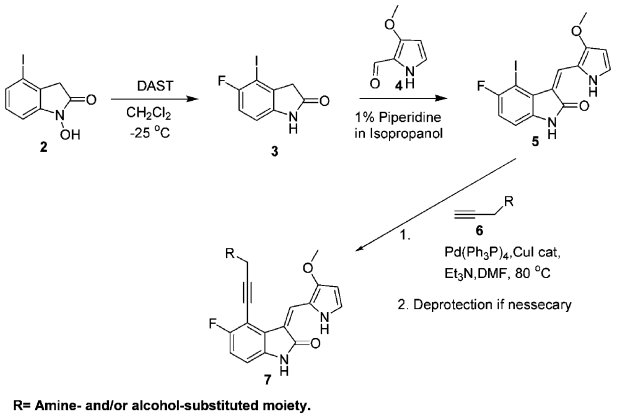
Table 1. CDK2/Cyclin E and tumor cell growth inhibition by 4-alkynyl oxindoles **7**

Compd	C-4 Substituent	CDK2/ Cyclin E IC_{50} (nM)[a]	MDA MB 435 IC_{50} (nM)[a]	SW480 IC_{50} (nM)[a]	Compd	C-4 Substituent	CDK2/ Cyclin E IC_{50} (nM)[a]	MDA MB 435 IC_{50} (nM)[a]	SW480 IC_{50} (nM)[a]
1b	H	847	>30,000	—[b]	**7j**		95	1270	967
6	I	1000	>30,000	—[b]	**7k**		9[c]	300	210
7a		4[c]	490	150	**7l**		7[c]	358	140
7b		21	110	130	**7m**		18	1070	549
7c		26	190	400	**7n**		45	2625	1185
7d		4[c]	130	110	**7o**		3[c]	400	140
7e		3[c]	320	270	**7p**		4[c]	600	290
7f		4[c]	300	80	**7q**		6[c]	110	92
7g		5[c]	300	205	**7r**		2[c]	31	40
7h		8[c]	349	100	**7s**		4[c]	63	44
7i		21	1160	566	**7t**		5[c]	110	90

[a] IC_{50} values were determined by a single experiment run in duplicate.
[b] Not tested.
[c] Values at assays detection limit.

orders of magnitude. Analogues that were substituted on the propargyl heteroatom by a group larger than a methyl moiety where overall slightly less potent that their counterparts without such groups. For instance, analogues **7i** and **7j** were overall less potent than alkynyl oxindoles **7g** and **7 h.** Derivatives **7m** and **7n** were less potent than their respective des-methyl or methyl-substituted counterparts **7k** and **7l**.

The crystal structure of CDK2 complexed with derivative **7o** affords a rationale for the observed potency of our 4-alkynyl oxindoles (Fig. 2).[9] That structure shows that **7o** binds in the ATP pocket of CDK2 via four major hydrogen bonding interactions. As expected, three of these interactions involve the oxindole-3-methylpyrrole core of **7o** and residues Glu81-Leu83. The fourth interaction involves a hydrogen bond between the propargylic heteroatom on the C-4 alkynyl substituent and the Asp145 located at the ribofurano-side phosphate binding region of the CDK2 ATP pocket. Apparently it is this fourth hydrogen bond that is responsible for the increased potency of our 4-alkynyl derivatives. This correlates with the observation that substitution by large groups at the propargylic heteroatom results in inhibitors with reduced overall potency.



Scheme 1. General synthesis of 4-alkynyl oxindoles **7**.

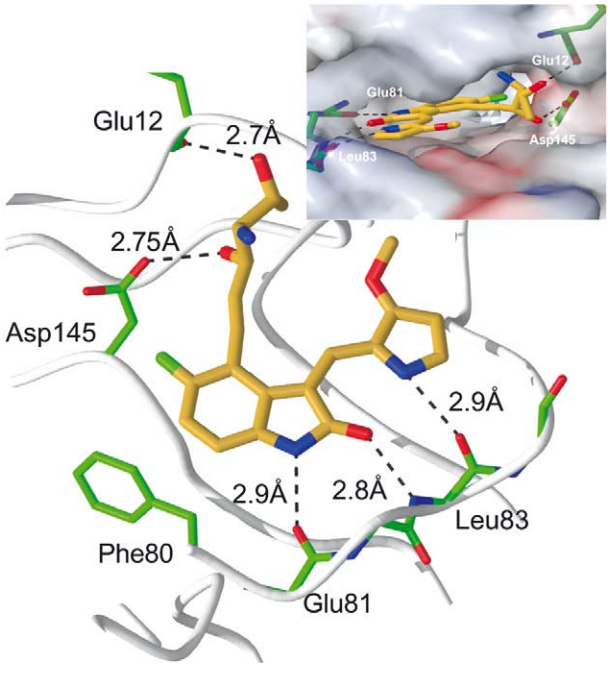


Figure 2. Crystal structure of inhibitor **7o** complexed with CDK2.

The CDK2-**7o** co-crystal structure also revealed that the terminal hydroxy group on the 4-alkynyl tether of **7o** makes a, seemingly unique for that particular inhibitor, fifth hydrogen bonding interaction with residue Glu12 from the protein backbone.

A representative from this class, compound **7q**, was evaluated further for its effect on the phosphorylation of retinoblastoma protein (Rb), one of the cellular substrates of CDK2. In these experiments SW480 cancer cells were incubated for 12 h or 24 h with **7q**. This resulted in a concentration dependent inhibition of the Rb phosphorylation (Fig. 3).[10]

The cell cycle analysis[11] of SW480 cells treated with **7q** showed that this 4-alkynyl-oxindole blocks cellular proliferation at the G1 and G2 phase and leads to a decrease in the percentage of cells in the S phase (Table 2). In addition, the treatment of these cells with **7q** also led to apoptosis as evident by the time- and dose-dependent increase of the subG0 fraction of cells (Table 2) and the DNA fragmentation experiment shown in Figure 4.[12]

The above biochemical effects correlate well with the cellular effects of other known CDK2 inhibitors.[13]

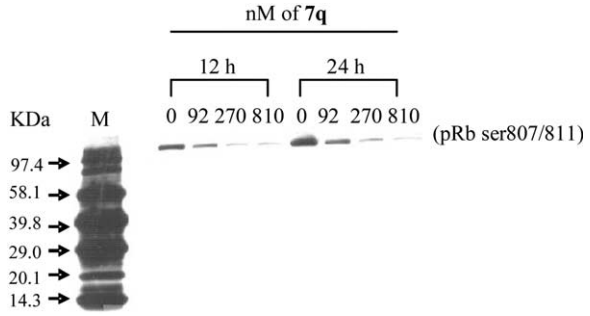


Figure 3. Inhibition of Rb phosporylation by **7q**.

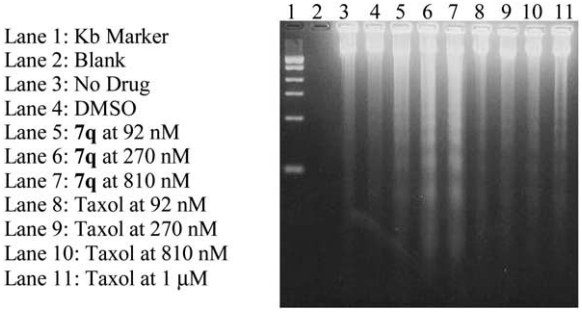


Figure 4. Electrophoretic gel mobility of DNA isolated from **7q**-treated SW480 cancer cells. Cells incubated with **7q** or Taxol, as control, for 24 h.

Table 2. FACS cell cycle analysis of SW480 cells treated with **7q**

Time of treatment	Concentration of **7q**		
	0 nM	92 nM	270 nM
4 h	G0-G1 49.25%	G0-G1 52.49%	G0-G1 43.90%
	G2-M 18.42%	G2-M 18.36%	G2-M 21.8%
	S 32.33%	S 29.14%	S 34.26%
	G2/G1[a] 1.94%	G2/G1[a] 1.95%	G2/G1[a] 1.95%
	%CV[b] 3.31%	%CV[b] 3.02%	%CV[b] 3.41%
	SubG0 13.29%	SubG0 11.17%	SubG0 14.47%
16 h	G0-G1 46.55%	G0-G1 55.84%	G0-G1 47.74%
	G2-M 14.84%	G2-M 15.72%	G2-M 38.44%
	S 38.61%	S 28.43%	S 13.82%
	G2/G1[a] 1.95%	G2/G1[a] 1.96%	G2/G1[a] 1.94%
	%CV[b] 3.29%	%CV[b] 3.22%	%CV[b] 3.27%
	SubG0 11.29%	SubG0 13.52%	SubG0 43.52%
24 h	G0-G1 52.41%	G0-G1 54.68%	G0-G1 50.00%
	G2-M 14.89%	G2-M 15.16%	G2-M 35.45%
	S 32.70%	S 30.15%	S 14.54%
	G2/G1[a] 1.95%	G2/G1[a] 1.96%	G2/G1[a] 1.94%
	%CV[b] 3.09%	%CV[b] 3.15%	%CV[b] 3.19%
	SubG0 11.85%	SubG0 11.67%	SubG0 46.86%

[a] Ratio between the G2 and G1 cellular fractions.
[b] Coefficient of variation.

In summary, the lead oxindole **1b** was transformed to a new series of potent inhibitors of CDK2. The potency optimization of **1b** was achieved by the introduction of propargyl and/or homopropargyl heteroatom substituted moieties at its C-4 position.

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7. For experimental details, see: (a) Chen. Y.; Dermatakis, A.; Luk, K.-C.; Liu, J.-J. *US Pat. 6,252,068 B1*. (b) Chen. Y.; Dermatakis, A.; Liu, J.-J.; Luk, K.-C. *US Patent 6,303,793 B1*.
8. Assay details have been disclosed in ref 5.
9. The crystals of CDK2-**7o** 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 and 0.1 M Ches pH 9. 2.5% β-mercaptoethanol was added to 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 the Brookhaven National Laboratories. Data was processed to 2.0 Å with the HKL package (Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997** *276*, 307) to an R-sym of 0.036. The structure was refined with REFMAC (Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr.* **1997**, *D53*, 240) to an R-factor/Rfree of 0.234/0.273. The crystal coordinates have been deposited in the PDB with the accession code 1R78.
10. Protocol for assessment of Rb phosphorylation in SW480 cells: SW480 (2.25×10^6 cells/mL) cells were treated with 0.1% DMSO (as the control) or oxindole CDK2 inhibitor dissolved in DMSO. Cells were treated with compound concentrations equivalent to the IC_{50}, IC_{90} & $3\times IC_{90}$ as determined in the MTT assay. Cells were exposed to drug for 4 h, 16 h, and 24 h time points. For immunoblotting, cell pellets were resuspended in PLC lysis buffer, sonicated briefly and debris sedimented by microcentrifugation at 15,000 rpm at 4 °C. The supernatant was mixed with 2X SDS sample buffer with 5% b-ME, and equal amounts of protein per lane loaded onto a 4–20% Tris Glycine gel. Proteins were resolved by electrophoresis, transferred to nitrocellulose membrane which was blocked in BSA blocking buffer (1% BSA, 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) overnight at 4 °C. The membrane was then incubated with diluted 1° antibody (NEB Ser807/811 phospho-Rb dilution 1:1000) in BSA blocking buffer for 1 h at room temperature. The membrane was washed for 30 min with buffer changes every 5 min with wash Buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) and then incubated with 2° antibody conjugated to HRP (anti-mouse IgG-HRP and anti-rabbit IgG-HRP both at 1:2000) in BLOTTO buffer (5% nonfat milk powder, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The wash procedure was repeated for another 30 min after which specific binding was detected with ECL (Amersham) folowed by exposure to a film.
11. For the cell cycle analysis experiments cells were fixed in

70% ethanol, centrifuged for 1 min at 3000*g* at 25 °C, washed once with PBS, treated with 1 mg/mL ribonuclease (Sigma Chemical Co.) for 15 min at 37 °C and stained with 50 mg/mL propidium iodide (Sigma Chemical Co.) for 30 min at room temperature. Flow cytometry analyses were performed on a Becton Dickinson FaCSCalibur using the Becton Dickinson Cell Quest program.

12. Protocol for the cell viability-apoptosis experiments: SW480 cells were grown in 40 mL of DMEM High Glucose + 10% heat inactivated FBS, (purchased from GIBCO/BRL, Gaithersburg MD) at 2.5×10^6 cells/150 cm^2 flask for SW480 cells. After 24 h of growth at 37 °C with 5% CO_2, cells were at 30% confluency. Subsequently, cells were treated with each drug at IC_{50}, IC_{90} and $3\times$ IC_{90} for 4 h or 24 h. For the negative control cells were treated with 40 μL of DMSO for 4 h or 24 h and for positive control cells were treated with Taxol at the IC_{50}, IC_{90}, $3\times$ IC_{90}, and 0.1 mM for 4 h or 24 h. For each sample cells were collected by transferring the growth medium to a 50 mL centrifuge tube, collecting the 5 mL wash with D-PBS-calcium and magnesium free (GIBCO/BRL, Gaithersburg, MD), and collecting the trypsinized cells (3 mL of Trypsin for 5 min at 37 °C). Cells were pelleted by centrifugation at 3000 rpm for 5 min at 4 °C. Cells were resuspended in 10 mL of D-PBS and pelleted as above. Cells were resuspended in pre-warmed lysis buffer (1.0 M NaCl, 50 mM Tris–HCl, pH 8.0, 0.5% SDS, and 10 mM EDTA) with Proteinase K added and lysed overnight (16–24 h) at 37 °C without shaking. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) with vigorous vortexing for 1 min. The phases were separated by centrifugation at 16,000*g* for 5 min at room temperature. The upper aqueous phase was removed and the extraction was repeated. The DNA was precipitated overnight by adding 2 volumes of 100% ethanol to the extract, vortexed briefly, and stored at −20 °C. The DNA was pelleted at 16,000*g* for 5 min at room temperature. The DNA pellet was dried and resuspended in 30 mL of TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The DNA samples were then treated with RNAse for 30 min at 37 °C. The DNA was resolved by electrophoresis (1.5% agarose, 0.5 mg/mL of ethidium bromide) in order to assess intranucleosomal DNA cleavage. Ethidium-stained DNA was visualized with UV light.

13. (a) Lane, M. E.; Yu, B.; Rice, A.; Lipson, K. E.; Liang, C.; Tang, C.; McMahon, G. *Cancer Res.* **2001**, *61*, 6170. (b) Kim, K.; Kimball, D.; Misra, R.; Rawlins, D.; Hunt, J.; Xiao, H. Y.; Lu, S.; Qian, L.; Han, W. C.; Shan, W.; Mitt, T.; Cai, Z. C.; Poss, M.; Zhu, H.; Sack, J.; Tokarski, J. S.; Chang, C. Y.; Pavletich, N.; Kamath, A.; Humphreys, W. A.; Marathe, P.; Bursurker, B.; Kellar, K.; Roongta, U.; Batorsky, B.; Mulheron, J.; Bol, D.; Fairchild, C.; Lee, F.; Webster, K. *J. Med. Chem.* **2002**, *45*, 3905.