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# Pyrazolobenzodiazepines: Part I. Synthesis and SAR of a potent class of kinase inhibitors

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ABSTRACT

A novel series of pyrazolobenzodiazepines **3** has been identified as potent inhibitors of cyclin-dependent kinase 2 (CDK2). Their synthesis and structure–activity relationships (SAR) are described. Representative compounds from this class reversibly inhibit CDK2 activity in vitro, and block cell cycle progression in human tumor cell lines. Further exploration has revealed that this class of compounds inhibits several kinases that play critical roles in cancer cell growth and division as well as tumor angiogenesis. Together, these properties suggest a compelling basis for their use as antitumor agents.

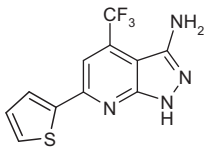


Cyclin dependent kinases are critical components utilized by cells in the progression through the cell cycle. Their aberrant control in a wide range tumor types has suggested a potential for therapeutic intervention and has been the subject of numerous studies and reviews.[1–4] While multiple avenues of exploration existed from the modulation of the various CDK activities, our initial excursion began with a focus on finding inhibitors of cyclin-dependent kinase 2 (CDK2).[5,6]

A high throughput screen identified the aminopyrazole compound **1** (Fig. 1) as a moderate CDK2 inhibitor (CDK2-cyclin E $IC_{50}$ = 2 μM) which had weak activity in the cell proliferation (MTT) assays (SW480, $IC_{50}$ = 6 μM; HCT116 $IC_{50}$ = 16 μM). As a precaution against false negatives in the high throughput screen, a differently formatted follow-up screen was conducted on structurally related substances in the screening inventory. One of the more interesting hits identified was compound **2**, a clonazepam analog prepared during an earlier program on valium-librium analogs.[7–11] This compound was found to be devoid of any CNS activity.

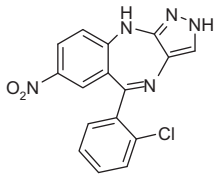
Despite the good enzymatic potency and interesting selectivity of compound **2**, it possessed modest cellular activity (SW480 $IC_{50}$ = 6 μM) and unattractive pharmacokinetic properties that prohibited further development. Herein, we describe the optimization of compound **2** with an emphasis on improving cellular potency and ultimately demonstrating in vivo efficacy. The initial SAR work was directed towards the exploration of the substitution on the two phenyl rings of **2**, with typical groups like methyl, nitro, acetyl and so on. In general, substitutions at positions other than at 7 or 2′ provided no improvements (data not shown).

The dramatic loss of activity by simple alkylation of the aminopyrazole nitrogens provided confirmation that these atoms were making key contributions to the binding. Further SAR exploration was then concentrated on manipulation of $R^1$, $R^2$, and $R^3$ groups in structure **3** (Fig. 2). This effort led to a series of novel, potent pyrazolobenzodiazepine derived multi kinase inhibitors, which had superior cellular activities compared to compound **2**.




**1**

CDK2-cyclin E 2 μM
CDK1-cyclin B 10 μM
CDK4-cyclin D1 > 30 μM
SW480 6 μM




**2**

CDK2-cyclin E 0.07 μM
CDK1-cyclin B 0.5 μM
CDK4-cyclin D1 10 μM
SW480 6 μM

**Figure 1.** Structure and biological activity of **1** and **2**.

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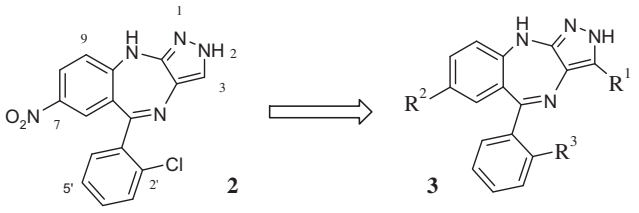
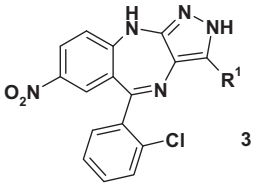


**Figure 2.** Optimization plan.

The general synthetic approach for the preparation of pyrazolobenzodiazepines **3** is shown in Scheme 1. The starting 1,4-benzodiazepines **4**, which can be easily prepared from substituted 2-amino-benzophenones according to the known methods,[7–11] were converted to the corresponding thiolactam **5** by the reaction with Lawesson's reagent. The thiolactam **5** was a more useful and adaptable intermediate for analog generation than the lactam **4**, due to its greater reactivity. When $R_1$ was hydrogen, final construction of the pyrazole ring system was accomplished by formylation [$Me_2NCH(OEt)_2$] of **5** to form enamine **6**, followed by reaction with hydrazine. In all other examples pyrazole ring formation required piperidine mediated coupling of **5** with an aldehyde ($R^1CHO$) to form **7**, followed by reaction of **7** with hydrazine to form the dihydropyrazole, and oxidation (air, DMSO, 150 °C) to afford the pyrazole, **3**.

The structure–activity relationship of $R^1$ was explored, keeping $R^2$ and $R^3$ fixed as shown in Table 1. These data show that only small hydrophobic groups were tolerated at $R^1$. Four analogs were more potent than **2** against CDK2 (**3a**, **3b**, **3h**, and **3j**).[6] The methyl (**3a**), 2-pyrrolyl (**3h**) and the 4-pyridinyl (**3j**) produced modest improvements in potency, while the isopropyl substitution (**3b**) provided the greatest improvement (7×). Hydroxymethyl (**3c**), trifluoromethyl (**3d**), amide (**3e**), phenyl (**3f**), phenylethyl (**3g**), and 3-pyridinyl (**3i**) analogs showed no improvement over hydrogen. In a SW480 cellular assay, three analogs (**3a**, **3b** and **3c**) were found to show potent inhibition of proliferation. Interestingly, the methyl analog **3a** is more potent than isopropyl analog **3b** although it's less active in the CDK2 assay.

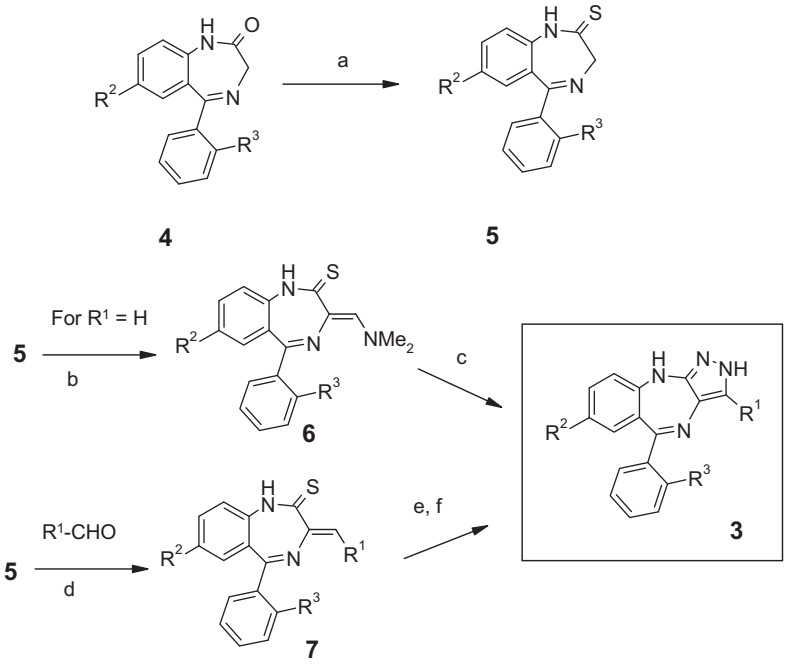
**Table 1**
Inhibition of CDK2 and cell proliferation ($IC_{50}$, μM) of analogs substituted at the pyrazole ring



| Compd | $R_1$ | CDK2[a,c] | SW480[b,c] |
|---|---|---|---|
| **2** | H | 0.070 | 6.00 |
| **3a** | Me | 0.035* | 0.16 |
| **3b** | *i*Pr | 0.010 | 0.64 |
| **3c** | $-CH_2OH$ | 0.238** | 0.24 |
| **3d** | $-CF_3$ | 0.232*** | N/A |
| **3e** | $-CONH_2$ | 0.258**** | N/A |
| **3f** | Ph | 0.210 | 18.00 |
| **3g** | $-CH_2CH_2Ph$ | 5.000 | 15.00 |
| **3h** | 2-Pyrrolyl | 0.020 | 5.30 |
| **3i** | 3-Pyridinyl | 0.300 | 26.00 |
| **3j** | 4-Pyridinyl | 0.050 | 12.00 |

[a] $IC_{50}$ values were determined with at least two replicates at each concentration except the following: *$n$ = 52, SD = 0.012; **$n$ = 2, SD = 0.031; ***$n$ = 3, SD = 0.039; ****$n$ = 2, SD = 0.053.
[b] Assay done once in duplicate wells.
[c] Assay details see Ref. 6.

An X-ray crystal structure of compound **3a** complexed with CDK2 is shown in Figure 3. The pyrazolobenzodiazepine core occupies the same site as the adenosine of ATP, and makes three critical H-bonds to the hinge region. $N^1$ of the pyrazole accepts a hydrogen bond from the backbone Leu83 NH. The pyrazole $N^2H$ forms a hydrogen bond with the backbone carbonyl of Glu81 and the diazepine $N^4H$ forms a hydrogen bond with the Leu83 carbonyl. The methyl substituent attached to the pyrazole ring is buried and



**Scheme 1.** Reagents and conditions: (a) Lawesson's Reagent, dimethoxyethane/75 °C; (b) $Me_2NCH(OEt)_2$/THF/rt; (c) Anhydrous hydrazine/$CH_2Cl_2$/rt; (d) $R^1CHO$/piperidine/dimethoxyethane/rt; (e) Hydrazine/DMSO/rt; (f) air oxidation, DMSO, 150 °C.

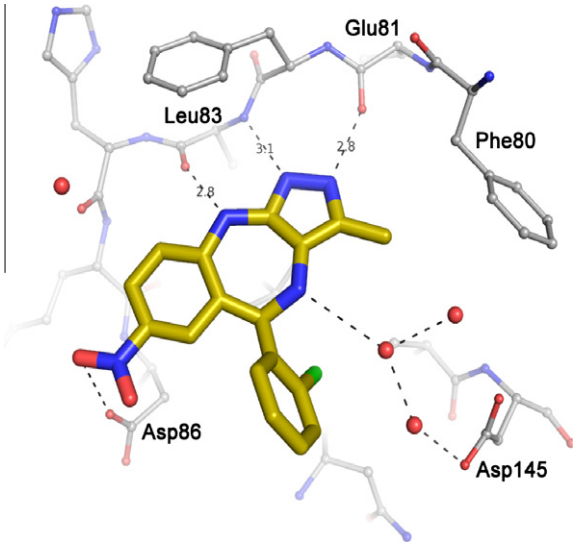
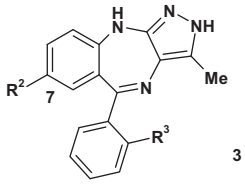



**Figure 3.** Crystal structure of compound **3a** bound to CDK2. Selected nearby protein residues are shown. Hydrogen bonding interactions with the protein are indicted as dashed black lines. The figure was prepared using PyMOL.

points towards the face of the gatekeeper phenylalanine (Phe80); this space could easily accommodate an isopropyl (most potent compound **3b**) or a small aromatic ring but not large substitutions. The chlorophenyl ring is oriented 80 degrees from the core, allowing the chlorine to point toward a mostly hydrophobic dimple created by Ala144, Leu134, and Asn132. The nitro substituent is solvent exposed and other water mediated contacts to the protein. Finally, the diazepine N is involved in a hydrogen bonding network to Asp145 via a series of bridging water molecules.[12]

We next focused on the modification of $R^2$ and $R^3$ groups on the aromatic rings with $R^1$ as methyl. The results are shown in Table 2.

**Table 2**
Inhibition of CDK2 and cell proliferation ($IC_{50}$, μM) of analogs with various $R^2$ and $R^3$ groups




3

| Compd | $R^2$ | $R^3$ | CDK2[a,c] | SW480[b,c] |
|---|---|---|---|---|
| **3a** | $NO_2$ | Cl | 0.035* | 0.16 |
| **3k** | H | H | 0.115 | 1.50 |
| **3l** | H | Cl | 0.105 | 3.80 |
| **3m** | H | F | 0.064 | 0.40 |
| **3n** | F | H | 0.093 | 2.20 |
| **3o** | F | F | 0.040 | 0.70 |
| **3p** | Cl | H | 0.490 | 5.50 |
| **3q** | Cl | Cl | 0.440 | 3.60 |
| **3r** | Cl | F | 0.190 | 3.00 |
| **3s** | CN | F | 0.016** | 0.11 |
| **3t** | OMe | Cl | 0.320 | 7.00 |
| **3u** | $NH_2$ | Cl | 0.088 | 0.50 |
| **3v** | AcNH | Cl | 0.038*** | 0.70 |
| **3w** | $NH_2CO$ | F | 0.017 | 0.21 |
| **3x** | $MeSO_2NH$– | Cl | 0.024 | 0.20 |
| **3y** | $Me_2NSO_2NH$– | Cl | 0.046 | 1.10 |

[a] $IC_{50}$ values were determined with at least two replicates at each concentration except the following: *$n$ = 52, SD = 0.012; **$n$ = 2, SD = 0.007; ***$n$ = 3, SD = 0.011.
[b] Assay done once in duplicate wells.
[c] Assay details see Ref. 6.

Replacement of either $R^2$ or both $R^2$ and $R^3$ with hydrogens resulted in a potency drop (**3k**, **3l** vs **3a**) of about threefold in the CDK2 assay and a 10- to 25-fold drop in the cellular assay. Other electron-withdrawing groups at the 7-position are also tolerated (**3s**, **3v**, **3w**, **3x**, and **3y**). On the other hand, electron-donating groups tended to decrease the potency in both the CDK2 and cellular assays (**3t** and **3u**). In general, fluoro was better than chloro in both assays (**3l** vs **3m**, **3q** vs **3r**).

A limited round of selectivity profiling was conducted with a few of the compounds to assess interactions with other kinase targets; the results are shown in Table 3. Compounds **3a**, **3s**, **3u** and **3v** all had weaker activity against CDK1, CDK4 and FAK.

We also tested this set of compounds against receptor tyrosine kinases involved in angiogenesis, such as KDR, FGFr, PDGFr and EGFr.[13–17] Of particular interest, compounds **3u** and **3v** showed good potency against KDR, FGFr, PDGFr and EGFr suggesting an additional therapeutic modality for this class of compounds (Table 4).

Compounds **3u** and **3v** were then tested in panel of five human tumor cell lines to confirm cellular activities. As shown in Table 5, both analogs demonstrated strong antiproliferative activities when tested in cells. The $IC_{50}$ values ranged from 0.3 to 1.5 μM. Subsequent evaluation in additional cell lines (data not shown) showed activity against a wide range of cancer cell types. However, more detailed studies on the effects of compound **3u** on the cell cycle (FACS analysis) revealed that its antiproliferative activities may not directly correlate with effects on CDK2.[18]

**Table 3**
Kinase inhibition profile of selected analogs ($IC_{50}$, μM)[a]

| Compd | CDK2[b] | CDK1[b] | CDK4[b] | FAK | Src[a] |
|---|---|---|---|---|---|
| **3a** | 0.035 | 0.310 | 0.252 | 6.900 | >60 |
| **3s** | 0.016 | 0.139 | 0.520 | 1.200 | 5.400 |
| **3u** | 0.088 | 0.382 | 0.303 | 0.150 | 1.840 |
| **3v** | 0.038 | 0.352 | 0.213 | 0.180 | 0.930 |

[a] $IC_{50}$ values were determined with at least two replicates at each concentration.
[b] Cyclin E1, D1, and A1 were used in the CDK assays as the CDK partners for CDK2, CDK4, and CDK1 respectively.

**Table 4**
Kinase inhibition profile of selected analogs ($IC_{50}$, μM)

| Compd | KDR[b] | FGFr[c] | PDGFr[d] | EGFr[e] |
|---|---|---|---|---|
| **3a** | 0.072 ± 0.015* | 0.100 | 0.083 | >10 |
| **3s** | 0.039 ± 0.008* | 0.067 | 0.051 | 10.900 |
| **3u** | 0.034 ± 0.006* | 0.050 ± 0.008* | 0.015 ± 0.002* | 0.989 |
| **3v** | 0.033 ± 0.005* | 0.045 ± 0.014* | 0.023 | 0.726 |

[a] $IC_{50}$ values were determined with at least two replicates at each concentration except the following: *$n$ = 2.
[b] With 300 μM ATP.
[c] With 10 μM ATP.
[d] With 2.3 μM ATP.
[e] With 0.5 μM ATP.

**Table 5**
Cellular activities of selected analogs ($IC_{50}$, μM)[a]

| Compd | H460a[b] | HCT116 | RKO | MDA-MB435 | SW480 |
|---|---|---|---|---|---|
| **3u** | 1.071 ($n$ = 6, SD = 0.210) | 1.018 ($n$ = 5, SD = 0.124) | 0.310 | 0.950 ($n$ = 2, SD = 0.050) | 0.500 |
| **3v** | 0.550 | 0.460 | 0.793 | 0.794 ($n$ = 3, SD = 0.074) | 0.701 |

[a] Values represent the mean of duplicates wells.
[b] The tissue origins are: lung: H460a; colon: HCT116, RKO, SW480; breast: MDA-MB435.

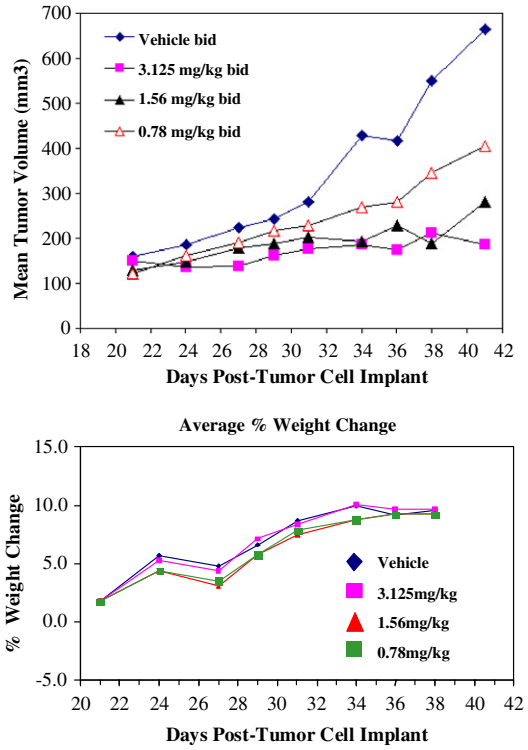



**Figure 4.** Efficacy and body weight changes in SW480 tumor-bearing nude mice treated with **3u** by oral administration. Compound was administered *per os* (po) using a 1-cc syringe and 18-gauge gavage needle (0.2 ml/animal; all groups were treated twice-daily (b.i.d.), 8 h apart, 7x/week for a total of 20 days. Treatments ended on day 41 post-implant.

Based on its superior overall profile in the kinase and cellular assays, and PK properties,[18] compound **3u** was selected for in vivo antitumor studies. Compound **3u** was administered orally for 20 days to tumor-bearing mice (SW480) at doses of 3.125, 1.56 and 0.78 mg/kg (b.i.d.). The compound was well tolerated, with no overt signs of drug related toxicity (ca. 9% body weight gain over the course of the study) and produced significant inhibition of tumor growth (Fig. 4). At the highest dose, 3.125 mg/kg, tumor growth inhibition was 93% (%T/C = 7, $p \leqslant 0.001$), while the mid dose (1.56 mg/kg) produced 70% tumor growth inhibition (%T/C = 30, $p = 0.021$). Compound **3u** has also shown significant antitumor activity in additional xenograft models (H460, LOX, LoVo and A549) as well as angiogenesis models.[17,18]

In summary, starting from pyrazolobenzodiazepine **2**, as a CDK2 inhibitor with modest cellular activities, we developed this novel template into a very interesting series of potent multikinase inhibitors. Further optimization of this pyrazolobenzodiazepine series with a focus on improving their dual activities as of anti-tumor and anti-angiogenesis agents will be described in subsequent publications.

## Acknowledgments

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## References and notes

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12. Crystals of the CDK2:**3a** complex were grown at 4 °C by the vapor diffusion method. CDK2(1–298) at 12 mg/mL was mixed with a five molar excess of compound and equilibrated against 10% PEG 3350, 0.2 M ammonium fluoride, 0.1 M Tris pH 8.5. 0.2% DTT 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 2.0 Å with the HKL package (Otwinowski, Z., Minor, W. *Methods Enzymol.* **1997**, *276*, 307). 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 0.196/0.243. The structure has been deposited to the PDB with the code 3LE6.
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