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Cyclin-Dependent Kinase 4 Inhibitors as a Treatment for Cancer. Part 2: Identification and Optimisation of Substituted 2,4-Bis Anilino Pyrimidines

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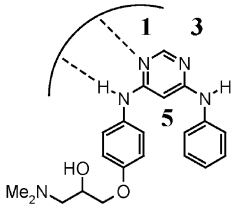
Abstract—Through chemical modification and X-ray crystallography we identified the 2,4-bis anilino pyrimidines as potent inhibitors of CDK4. Herein, we describe the optimisation of this series.

The cyclin-dependent kinases (CDKs) are important in controlling entry into and progression through the cell cycle.[1] They are a family of serine/threonine kinases whose activity is regulated at several levels. The binding of each CDK to a specific cyclin partner protein is required for activity. The synthesis and degradation of cyclins is tightly controlled such that their level fluctuates during the cell cycle. It is these fluctuating levels of the cyclin that cause first one, and then another member of the CDK family to become active during cell cycle progression. Inhibitory proteins also regulate CDK activity and these inhibitors need to be either sequestered or destroyed to allow the CDK enzyme to become fully activated. As cells respond to the presence of mitogens, the level of cyclin D1 increases and eventually triggers the activation of CDK4 and CDK6. These kinases phosphorylate the retinoblastoma tumour suppressor protein pRb, thereby abrogating its inhibition of members of the E2F family of transcription factors. This then triggers a programme of gene expression that results in entry into the S phase of the cell cycle.

There are now many examples where tumours contain multiple copies of the cyclin D1 gene or express abnormally high levels of the protein.[2] Similarly, many tumours have been described that contain mutations, deletions or silencing of the p16 or the pRb gene.[3,4] Finally, mutations of CDK4 itself have been described within melanoma patients, where the region of the enzyme that binds p16 has been mutated making the enzyme resistant to inhibition.[5] Together, these findings imply that the deregulation of the pRb pathway, and CDK4, is important in cancer progression. The inhibition of CDK4 may therefore be a valuable approach to treat tumours especially those that have lost the natural CDK4 inhibitor, p16.

A number of groups have identified CDK inhibitors.[6–15] As described in the previous paper[16] a series of 4,6-bis anilino pyrimidines were identified from a high-throughput screening campaign[17] as inhibitors of CDK4. During further investigation of this series, several X-ray structures were obtained with the structural surrogate CDK2 in order to improve our understanding of the compound's binding modes From these CDK2 complex structures, it was evident that the pyrimidine N1 acts as a hydrogen bond acceptor with the 6-aniline NH serving as a hydrogen bond donor.[16]



In contrast, it appeared the pyrimidine N3 was not involved in any specific interaction. This led us to

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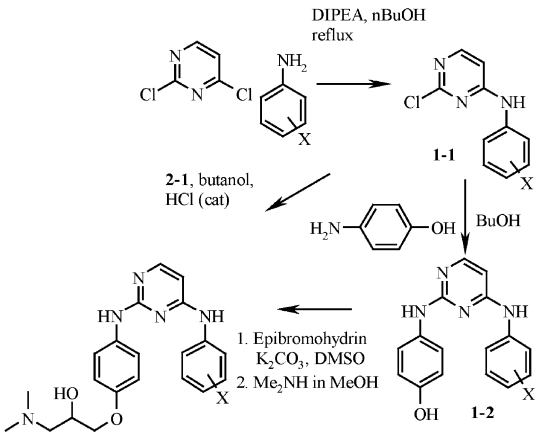
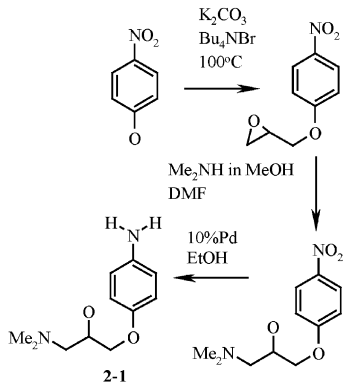
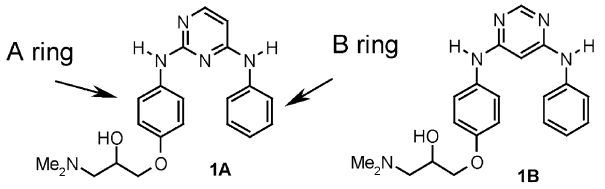


Scheme 1. Synthesis of 2,4-bis anilino pyrimidines.



Scheme 2. Synthesis of **2-1**.

speculate about the likely utility of a 2,4-bis anilino pyrimidine (i.e., where the pyrimidine N3 is moved to the equivalent of the 5 position). We considered the possibility that the pyrimidine 5H of the 4,6 series may contribute to the twisting of the aniline rings out of the plane of the pyrimidine in the bioactive conformation. If the twisted conformation were crucial to the activity then the 2,4 series may prove to be less potent. Despite these concerns, we initiated chemistry around the 2,4-bis anilino pyrimidines.

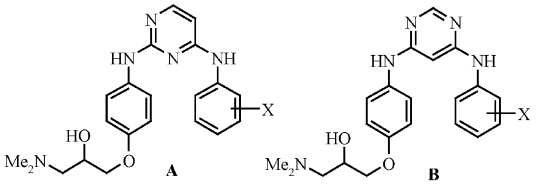


The first compound prepared was the 2,4-bis anilino pyrimidine **1A** (IC_{50} 2 μM); this compared favourably with the analogous 4,6-bis anilino pyrimidine **1B** (IC_{50} 33 μM) and, therefore, we decided to explore the series further. Our aim was to confirm the relative binding modes, and explore the possibility of overlapping SAR between the two series, and then to utilise this information to obtain improvements in potency.

The route used for the preparation of the 2,4-bis anilino pyrimidines is shown in Scheme 1. The 2,4-dichloropyrimidine was reacted with the appropriate aniline to introduce ring B. In this step, butanol was predominately the solvent of choice although NMP was occasionally employed for poorly nucleophilic anilines. Conversion of intermediate **1-1** to the final product could be achieved in a stepwise fashion or, more convergently, by reaction with the functionalised aniline **2-1** (Scheme 2) in butanol with acid catalysis to yield the product in one step.

With the synthetic route established we were able to explore the SAR and turned our attention to investigating the effects of substitution in ring B. From the initial compounds prepared, results indicated that the 2,4 substituted pyrimidines were generally more potent

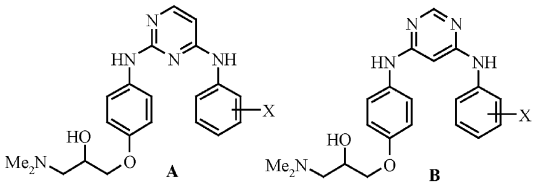
Table 1. Structures and enzyme activity for the 2,4-bis anilino pyrimidines compared to the 4,6 bis anilino pyrimidines



Compd	X	A		B	
		CDK4 IC_{50} (μM)[a]	CDK2 IC_{50} (μM)[a]	CDK4 IC_{50} (μM)[a]	CDK2 IC_{50} (μM[a])
1	All H	2	34	33	>100
2	2-F	1	7	27	>100
3	2-Cl	1	6	8	95
4	2-Br	1	7	4	28
5	2-CN	2	4	***	***
6	2-OCH_3	19	15	23	85
7	3-Cl	2	5	***	***
8	4-Cl	1	18	41	>100
9	4-OCH_3	2	36	57	>100
10	4-CH_3	2	26	25	>100

[a]Values are means of at least two determinations. The assay-to-assay variation was generally ±2-fold based on the results of a standard compound.

Table 2. Structures and enzyme activity for the disubstituted 2,4-bis anilino pyrimidines compared to the disubstituted 4,6-bis anilino pyrimidines



Compd	X	A		B	
		CDK4 IC_{50} (μM)[a]	CDK2 IC_{50} (μM)[a]	CDK4 IC_{50} (μM)[a]	CDK2 IC_{50} (μM)[a]
3	2-Cl	1	6	8	95
11	2-Cl, 5-Cl	0.8	1	1	22
12	2-F, 5-CH_3	0.7	2	4	81
13	2-Cl, 5-CH_3	2	5	2	22
14	2-F, 5-CF_3	0.6	1	6	35

[a]Values are means of at least two determinations. The assay-to-assay variation was generally ±2-fold based on the results of a standard compound.

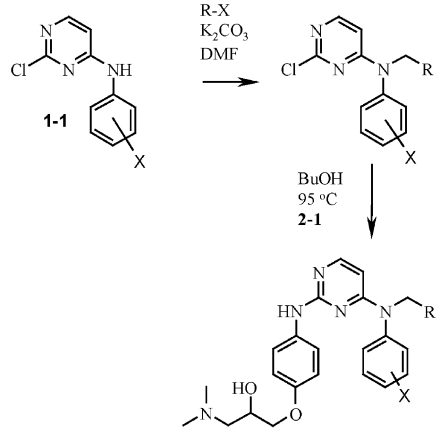
than the 4,6-equivalent with improvements in IC_{50} ~10-fold (Table 1) and in selectivity (for CDK4 over CDK2) generally being ~10-fold. There appeared to be little variation in potency over the range of substituents in the 2,4 compounds chosen **1A**–**10A**, unlike their 4,6 counterparts, **1B**–**10B**. Interestingly, some substituents did not conform to the general activity trend. For example, the isomeric 2-bromo inhibitors **4A** and **4B** have virtually identical potencies. Also of note is compound **6A**, substituted with methoxy at the 2-position, as it is markedly less potent as well as having no selectivity versus CDK2.

As disubstitution had proved a successful way to improve potency in the 4,6 series, we decided to investigate the effects in the 2,4 series (Table 2). In contrast to the 4,6 series, the disubstituted compounds showed no substantial improvement in potency over their monosubstituted analogues. For example, the 2,5-dichloro analogue **11A** showed an IC_{50} similar to that for the simple 2-chloro **3A**. Note also that there has been an apparent decline in selectivity with the disubstituted compounds, CDK4 versus CDK2. The reason for this reduction in selectivity remains unclear.

The X-ray structure[18] for the complex of CDK2 with the 2,4 disubstituted compound **11A** (Fig. 1) was obtained. The binding mode of the 2,4 series is the same as found earlier for the 4,6 series,[16] utilizing a single pyrimidine nitrogen and the A-ring aniline NH to form hydrogen bonds to the protein backbone. However, the tilt of the B-ring with respect to the pyrimidine was found to vary substantially between different enzyme/inhibitor complex structures both within and between the two series. This may go some way to explaining why the SAR of the two series is difficult to understand and do not appear to correlate.

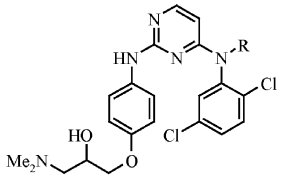
Having established that *N*-alkylation improved potency in the 4,6 series, we decided to look at the effects within the 2,4 series since the X-ray structure of the CDK2–**11A** complex suggested displacement of the water was still possible. The alkylated compounds were obtained by reaction of intermediate **1-1** with the appropriate alkyl bromide in DMF with potassium carbonate as base (Scheme 3). This was then reacted with intermediate **2-1**, by heating at 95 °C in butanol overnight.

Table 3 shows the activity results for the alkylated compounds. The improvement in potency was less



Scheme 3. Synthesis of the alkylated 2,4-bis anilino pyrimidines.

Table 3. Structures and enzyme activity for the alkylated 2,4-bis anilino pyrimidines



Compd	CDK4 IC_{50} (μM)[a]	CDK2 IC_{50} (μM)[a]	R
11	0.8	1	H
15	0.1	0.7	CH_2CN
16	0.2	2	CH_2CCH
17	0.3	3	CH_2CH_2CN
18	1	>10	CH_2Ph
19	0.2	12	$(CH_2)_3CF_3$

[a]Values are means of at least two determinations. The assay-to-assay variation was generally ±2-fold based on the results of a standard compound.

marked than we had expected (~10-fold at best) by comparison to the 4,6 series.[16] In addition, the different *N*-alkyl groups showed only modest variation in potency. However, in keeping with the findings in the 4,6 series,[16] the cyanomethyl **15** still delivered good potency but now stood out less from the acetylene **16**. A drop in potency was still observed as the chain was lengthened by one carbon unit **17**, but, bulky substituents (**18** and **19**) were better tolerated in this series. It should also be noted that the *N*-alkylation also gave improved selectivity with respect to CDK2 compared to the disubstituted compounds (Table 2), possibly due to a reduced freedom to rotate around the pyrimidine–4N bond.

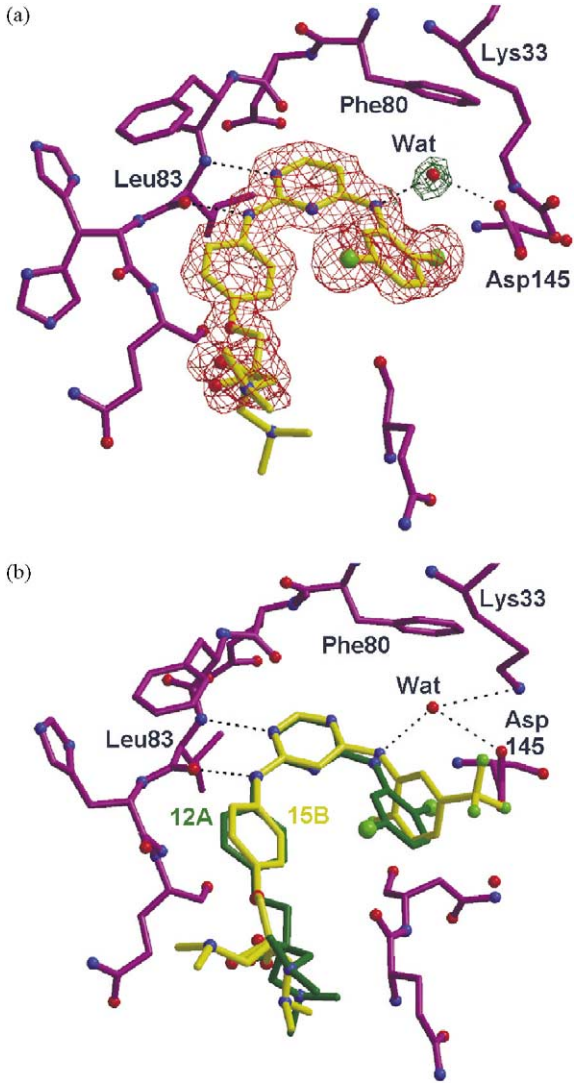


Figure 1. (a) X-ray structure of CDK2 complexed with **11A** showing final Fo-Fc difference electron density calculated for a model from which **11A** was omitted (red, contoured at 2.5σ) for the bound ligand in the ATP-binding pocket. Selected nearby protein residues are shown. Hydrogen bonding interactions with the protein are indicated and are as expected. Ring B is well defined. The solubilising substituent on ring A has been modelled as a racemic mixture and is only partially ordered. The bridging water molecule between the 4-anilino NH and Asp145 is shown in green density. Other solvent molecules have been omitted for clarity. Note that Lys33, which normally forms a salt bridge with Asp145, has electron density (not shown for clarity) that indicates it has been covalently modified: it is carboxylated. (b) Superposition (based on Cα positions of CDK residues 80–86) of **11A** (green) and **14B**[16] (yellow) suggesting that, as for **14B**, *N*-alkylation to displace the water molecule may also be a route to improving potency in **11A**. Figures 1 and 2 were prepared using Bobscript and Raster3D.[27–29]

Looking at the X-ray structure of CDK2 with **11A** (Fig. 1), it was evident that the part of the binding site local to the *N*-alkyl substituent could also be reached by placing a group at the 5-position of the pyrimidine. For this reason, we decided to investigate substitution at the 5-position. Compounds were prepared from the commercially available 5-substituted uracils by conversion to the corresponding dichloropyrimidines using phosphorus oxychloride and phosphorus pentachloride at reflux. The synthetic route detailed in Scheme 2 could then be followed (Table 4).

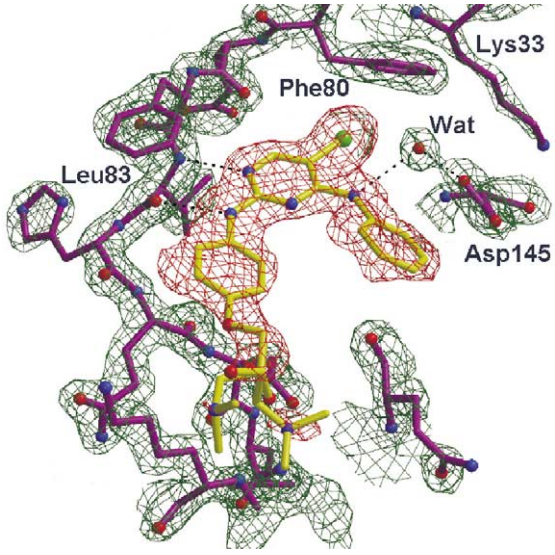
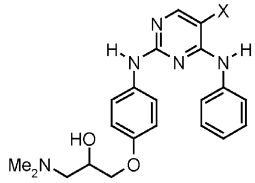


Figure 2. Electron density at the ATP-binding site in the X-ray structure of CDK2 (2Fo-Fc; contoured at 1.5σ, green density) bound to **23** (Fo-Fc; contoured at 2.5σ, red density). Apart from the solubilising group, which is modelled as the racemate, the ligand is very well defined. The bridging water is not displaced in this structure and the 5-bromo substituent stacks against Phe80.

Table 4. Structures and enzyme activity for the 5-substituted 2,4-bis anilino pyrimidines



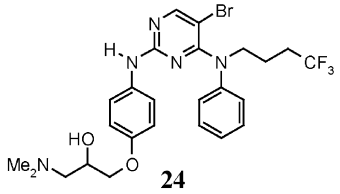
Compd	CDK4 IC_{50} (μM)[a]	CDK2 IC_{50} (μM)[a]	X
1	2	34	H
20	0.8	8	CH_3
21	0.3	>10	F
22	0.1	0.5	Cl
23	0.1	0.3	Br

[a]Values are means of at least two determinations. The assay-to-assay variation was generally ±2-fold based on the results of a standard compound.

Initially, we explored multi-atom substituents at the 5-position attempting to pick up the interactions that *N*-alkylation provided. However we were unable to improve activity. Looking more broadly, we found that halogens, especially chloro **22** and bromo **23**, improved the potency against both CDK2 and CDK4. However, this improvement was not accompanied by the good selectivity that had been observed for the *N*-alkylated compounds (Table 2). These results suggested that the smaller 5-substituents were not occupying the same part of the binding site as the *N*-alkyl group. The X-ray structure of CDK2 in complex with **23** was determined, and, as anticipated, the bridging water molecule remained in place and the bromo substituent packed against Phe80 (Fig. 2).

Increasing the bulk of the 5-substituent lead to increased activity, possibly because the bioactive ring B orientation becomes a more favourable small molecule conformation (**1** cf. **20**). Increasing the 5-substituent polarisability also leads to increased activity, possibly due to improved interactions with the π cloud of the Phe80 aromatic ring (**21**–**23**).

This led us to consider whether combining 5-substitution and *N*-alkylation might provide compounds that are both potent and selective. These compounds were prepared according to Scheme 3 starting with the appropriately substituted dichloropyrimidine. Our work in this area led us to compound **24** that had an IC_{50} for CDK4 of 0.01 μM. The combination of 5- substitution and *N*-alkylation gave not only good potency versus CDK4 but also good selectivity (**24**, CDK2 IC_{50} 0.2 μM).



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In conclusion, we have demonstrated that using our understanding of the binding mode, we were able to convert our initial 4,6-bis anilino pyrimidine series to the 2,4-bis anilino pyrimidines. The 2,4-bis anilino pyrimidines are also potent and selective inhibitors of CDK4. However potency and SAR are not mirrored in the two series which we believe is due to conformational variability in particular of the B ring, and the scope for different substitution in the pyrimidine core.

Acknowledgements

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References and Notes

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18. Protein and crystals were obtained according to established procedures[19,20] Diffraction data were collected on beamline 14.3 at ESRF, Grenoble, at 100 K. Data processing, data reduction and structure solution by molecular replacement were carried out using programs from the CCP4 suite.[21] Compounds **11A** and **23** were modelled as racemic mixtures of the chiral alcohol using InsightII,[22] and modelled into electron density using QUANTA.[23] The protein complex model was refined using CNX,[24] and final structures[25,26] have been deposited in the Protein Data Bank with deposition codes 1h01 and 1h08 together with structure factors and detailed experimental conditions.
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22. InsightII, Accelrys.
23. Quanta2000, Accelrys.
24. CNX version 2000.1, Accelrys.
25. Crystallographic statistics for **11A** are: space group $P2_12_12_1$, unit cell 53.1, 70.7, 72.5 Å, resolution 1.8 Å, 24527

unique reflections from 58,865 observations give 94% completeness with $R_{merge} = 3.3\%$ and mean $I/\sigma(I)$ of 14.The final model containing 2247 protein, 244 water, 12 glycerol and 37 inhibitor atoms has an R-factor of 19% (R_{free} using 5% of data is 22%). Mean temperature factor for protein is 17.5 and for ligand is 17.3 Å^2.
26. Crystallographic statistics for **23** are: space group $P2_12_12_1$, unit cell 53.2, 71.9, 72.0 Å, resolution 1.8 Å, 26,090 unique reflections from 106,969 observations give 99.5% completeness with $R_{merge} = 5.7\%$ and mean $I/\sigma(I)$ of 18. The final model containing 2247 protein, 181 water, six glycerol and 36 inhibitor atoms has an R-factor of 21% (R_{free} using 5% of data is 24%). Mean temperature factor for protein is 32.7 and for ligand is 40.8 Å^2.
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