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2,6,9-TRISUBSTITUTED PURINES : OPTIMIZATION TOWARDS HIGHLY POTENT AND SELECTIVE CDK1 INHIBITORS

Patricia Imbach* [+], Hans-Georg Capraro[+], Pascal Furet[+ ‡], Helmut Mett[+], Thomas Meyer[+] and Jürg Zimmermann [+]

Novartis Pharmaceuticals, Therapeutic Area Oncology, Novartis Limited, CH-4002 Basel, Switzerland

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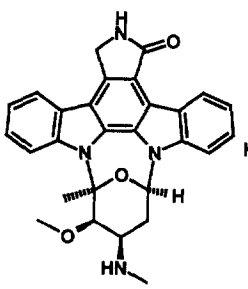
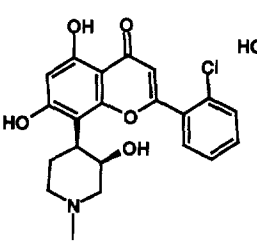
Abstract: Novel 2,6,9-substituted purine derivatives represent a class of potent and selective inhibitors of CDK1/cyclinB. The synthesis, SAR and biological profile of selected compounds are described.

INTRODUCTION. The cell division cycle typically includes two major phases: the S-phase, during which the DNA is replicated, and the M-phase, during which the cell divides. During the first transition phase, G1, the cell prepares for the replication of its DNA whereas in the second transition phase G2, the cell gets ready for mitosis. Progression through the cell division cycle is driven by a family of protein kinases, the so-called cyclin dependent kinases (CDKs).[1-3] The CDKs are activated through binding to a family of regulatory proteins called cyclins and phosphorylation on a specific threonine residue.[4-8] Multiple genetic alterations in neoplastic cells affect the regulation of the cell cycle machinery. As a result, there is a considerable interest in restoring normal cell cycle control in tumor cells using therapeutic approaches such as gene therapy or small molecular inhibitors of CDK's.

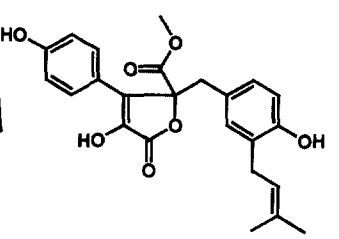
At least four types of CDK inhibitors have been described so far. Staurosporine is a powerful but non-specific kinase inhibitor.[9-10] Flavopyridole L86-8275 and butyrolactone I have been described recently as selective inhibitors of CDK1 and CDK2.[11-12] The fourth structural class are purine derivatives. 6-Benzylamino-2-(2-hydroxyethylamino)-9-methylpurine (**1**, renamed olomoucine), was found to be a moderately active, but - in contrast to staurosporine - specific inhibitor of CDK1.[13-14]



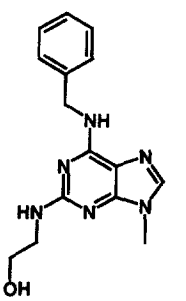
Staurosporine



flavonoid L86-8275



butyrolactone-1



Olomoucine, **1**

[+] *Oncology Research* [‡] *Molecular Modelling* * *e-mail: patricia.imbach@pharma.novartis.com; FAX: ++41 61 696 62 46*

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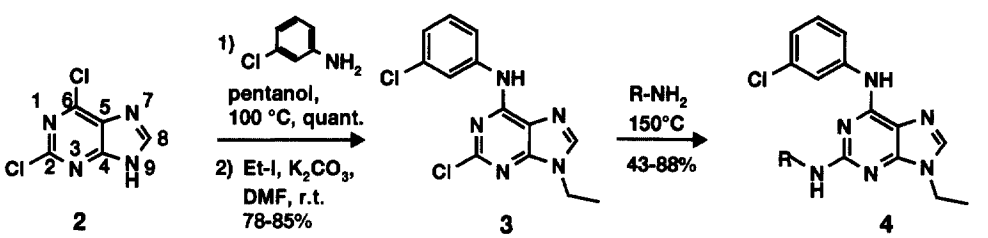
The crystal structure of the human cyclin A/CDK2/ATP complex was resolved in 1993.[15] Shortly thereafter, the crystal structures of CDK2 with the inhibitor molecules flavonoid L868276,[16a] and olomoucine[16b] were published. Based on this information, we developed in house a model of the binding mode of olomoucine in the ATP binding pocket, which allowed in a first step the optimization of olomoucine **1** towards highest enzymatic activities.[17] Also, the purine nucleus is well suitable for a combinatorial approach in order to generate a high number of derivatives, and several reports on this matter have been published recently.[18-23] However, by combining our knowledge from x-ray data, molecular modeling and medicinal chemistry, we were able to prepare new and highly active olomoucine derivatives by synthesizing only a small number of designed derivatives.

In this Letter we enclose our preliminary findings on a series of new 2,6,9-trisubstituted purines which are capable of inhibiting cyclin dependent kinase CDK1 at low nanomolar concentrations. Potential clinical applications of these inhibitors include their use as anticancer agents. The combination of CDK1 inhibition as well as the different effect on normal versus tumor cells (specificity) is expected to translate not only into antitumor efficacy (tumor regression) but also in good tolerability of the compounds.

CHEMISTRY. The straight forward, 3-step synthesis of C-(2), C-(6) and N-(9) trisubstituted purines was started from commercially available 2,6-dichloropurine (2,6-DCP) **2**. As outlined in scheme 1, 2,6-DCP was reacted with an aniline such as 3-chloroaniline in pentanol to obtain quantitatively the C-(6) substituted compound; followed by N-(9)-alkylation to obtain compound **3**.[24] To avoid N-(7) alkylation, it was necessary to substitute the C-(6) chlorine first. Intermediate **3** was further reacted with the corresponding amine; neat if suitable, or by using a high boiling solvent such as DMSO, *t*-butanol or diglyme to yield compound **4**.[25] The yields of these non-optimized reactions vary between 43-88%.

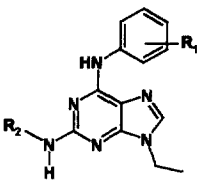
Scheme 1: General synthesis of 2,6,9-trisubstituted Purine Derivatives

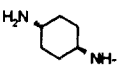
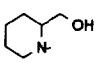
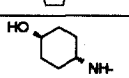


RESULTS AND DISCUSSION. Olomoucine **1** is a moderately active (IC_{50} of 4.5 μM on CDK1) but very selective CDK1 inhibitor. Thus, attempts were undertaken to increase the potency of this lead without loosing its high selectivity. From a first series of derivatives (table 1), some general rules, based on enzymatic data, could be drawn. The window for a substitution at N-9 proved to be rather small, with N-9-ethyl being about equipotent to isopropyl; and $-CH_2CH_3 > CH_3 > H$ (data not shown). The first steps of optimization resulted in the *m*-Cl-anilino substituted compound **5** with an IC_{50} of 330 nM on CDK1, which is an improvment of a factor 14 compared to the parent compound and other benzylamino derivatives of olomoucine **1**[26] (see table 1).

Subsequently, the substitution pattern of the aniline ring as well as the C(2)-substitution were optimized, keeping the other parts of the molecule constant.

Table 1. Optimization of of 2,6,9-trisubstituted Purine Derivatives



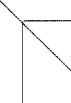
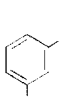
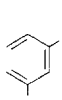
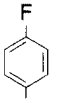
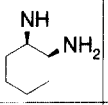
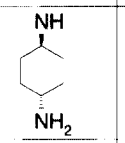
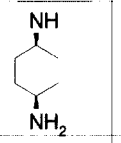
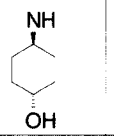
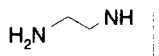
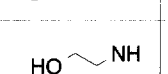
Cpd.No.	R_1	R_2	CDK1 [b] IC_{50}[μM]	T24 [i] IC_{50}[μM]	FAB-MS $[M+H]^+$	mp [°C]
5	3-Cl	$-CH_2CH_2OH$	0.33	17.5	333	99-102
6	3-Cl	$-CH_2CH_2NH_2$	0.08	3.16	332	103
7[a]	4-F	$-CH_2CH_2NH_2$	0.45	8.3	316	>250
8[a]	3-CN	$-CH_2CH_2NH_2$	0.56	5.24	323	>175 dec.
9	3-OCH_3	$-CH_2CH_2NH_2$	0.55	9.9	328	oil
10[a]	3-CF_3	$-CH_2CH_2NH_2$	0.7	6.08	366	>250 dec.
11[a]	3-F	$-CH_2CH_2NH_2$	0.60	6.5	316	>250 dec.
12[b] (1-R, 2-R)	3-Cl		0.11	1.35	386	152
13[c]	3-Cl		0.025	0.48	386	106-110
14[d]	3-Cl		0.12	1.50	386	foam
15	3-Cl		0.039	0.54	386	193-194
16[e]	3-Cl		0.039	3.65	387	184
17[f]	3-Cl		0.08	6.71	373	164-165
18[g]	3-Cl		0.041	2.35	372	128
19	3-Cl		0.03	0.83	387	117

[a] submitted as HCl salt [b] mixture of enantiomers (trans), prepared from [1R,2R]diaminocyclohexane [c] active cis enantiomer; absolute configuration not determined [d] mixture of enantiomers (cis) [e] mixture of enantiomers [f] prepared from D-prolinol [g] prepared from D-prolinamid via reduction with $LiAlH_4$
[h] experimental details of the enzyme purification and assay are described in references 27-31 [i] experimental details of the assay are described in reference 32

The best enzymatic activity amongst the different aniline substituents was found with the 3-chloro substituted compound **6**, followed by the 4-fluoro-compound **7** (table 1). No further improvements were achieved with 3-CN, 3-OCH_3, 3-CF_3 or 3-F substitutions on the aniline ring (cpds. **8**-**11**).

The first compound with an IC_{50} below 100nM was **6** where the C(2)-hydroxy side chain had been replaced through an amino group. Further improvement of enzymatic activity was obtained when the rotational degrees of freedom were restricted at the C(2)-position. Diaminocyclohexyl-, piperidine- and pyrrolidine side chains resulted in enzymatic activity below 100 nM (table 1, compounds **12**-**19**). Especially the diaminocyclohexane derivatives **13** and **15** as well as the *trans*-4-hydroxy-aminocyclohexane compound **19** showed - besides excellent enzymatic activity in the nanomolar range - very promissing antiproliferative activities on the bladder carcinoma cell T_{24} with an IC_{50} below 1 μM. In a further optimization sequence, the syntheses of a combination of the most active substituents at C(2) and C(6) were performed (table 2).

Table 2 **C-2/C-6 Further optimization (substituent at N-9: $-CH_2-CH_3$): IC_{50} [μM]on CDK1**

C-2 \ C-6						
	0.031	0.31	0.25	0.15	0.23	0.35
	0.039	0.14	0.15	0.031	0.16	0.085
	0.14	0.29	0.35	0.1	0.34	0.35
	0.03	0.028	0.032	0.04	0.09	0.028
	0.08	0.7	0.6	0.45	0.55	0.56
	0.33	n.a.	0.44	0.38	1.0 [1)]	n.a.

N.B.: *) HO-$(CH_2)_3$- at C-2

From this series of compounds we can clearly see the high enzymatic potency of all combinations of a C(2)- 1,4-*trans*-hydroxy-cyclohexylamine substitution with an aniline at C(6); all these compunds have IC_{50} values in the low namolar range (28-90 nM). Other potent combinations are found with *trans* and *cis* 1,4-diaminocyclohexylamine. Concerning the anilines, the best enzymatic activities are obtained with a *p*-fluoro, *p*-trifluoromethyl or *m*-chloro substitution respectively.

Some of the most potent compounds are being further evaluated in regard of their selectivity against other kinases (table 3). Selectivity against PKCα, PKA and EGF was achieved by a factor 10-100.

Table 3. Selectivity Profile

Cpd.Nr.	CDK1 IC50[μM]	PKC-α[a)] IC50[μM]	PKA[b)] IC50[μM]	EGF[c)] IC50[μM]
6	0.08	31.5	180	>100
8	0.56	78	>500	>10
10	0.7	50	500	>10
12	0.11	30	335	2
13	0.025	6.1	125	>10
14	0.12	13.5	150	5.1
15	0.039	6.0	130	5.3
17	0.08	38.5	>500	n.a.

[a,b,c)] purification of protein kinases and in vitro enzyme tests are described in reference 33

Further biological evaluation *in vitro* and *in vivo* of selected purine derivatives is ongoing and will be reported elsewhere.

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[24] Kjellberg, J.; Johansson, N. G. *Nucleosides & Nucleotides* **1989**, *8(2)*, 225-256.
[25] A typical three step synthesis is done as follows: (A) 1.4 ml (13 mmol) of 3-chloroaniline are added to a suspension of 650 mg (3.44 mmol) of 2,6-dichloro-purine in 5 ml of 1-pentanol. The reaction mixture is stirred at 100 °C (bath temperature) for 3 hours. After cooling to rt, the mixture is diluted with isopropanol and stirred at 10 °C for 90 minutes. The precipitate is filtered off and rinsed with isopropanol and diethyl ether. The crystals are partitioned between 50 ml of 2 N NaOH soln., 100 ml of water and 700 ml of ethyl acetate. The aqueous phase is subsequently extracted with ethyl acetate (2x). The combined organic extracts are washed with water, satd NaCl soln. and dried (Na_2SO_4). After filtration and concentration, the crude product is stirred with diethyl ether and the crystals are dried at 50 °C under an HV to obtain 2-Chloro-6-(3-chlorophenylamino)-purine (923 mg, 96%).(B) 676 mg (2.41 mmol) of 2-chloro-6-(3-chlorophenylamino)-purine are dissolved in 10 ml of abs. DMF by gentle heating. 375 mg (2.71 mmol) of K_2CO_3, followed by 0.97 ml (12.01 mmol) of ethyl iodide are added at rt. The reaction mixture is stirred at rt for 2 h. Upon completion, the reaction mixture is poured onto ice/water (60 ml) and stirred for 10 minutes. The mixture is extracted with ethyl acetate (3x), the combined organic extracts are washed with water, satd NaCl solution and dried ($MgSO_4$). After filtration and concentration, the resulting crude product is purified by crystallization from diethyl ether/hexane to obtain 2-Chloro-6-(3-chlorophenylamino)-9-ethyl-9*H*-purine (630 mg, 85%); m.p. 127-128°C.(C) 250 mg (0.81 mmol) of 2-chloro-6-(3-chlorophenylamino)-9-ethyl-9*H*-purine are dissolved in 5.8 ml (97 mmol) of ethylenediamine and the solution is heated under reflux for 3 hours (150 °C). After cooling to rt, the reaction mixture is taken up in ethyl acetate (250 ml) and extracted with water (150 ml). The aqueous phase is extracted with ethyl acetate (2x) and the combined organic extracts are washed successively with saturated $NaHCO_3$ solution, water, satd NaCl solution, dried ($MgSO_4$). After filtration and concentration, the crude product is recrystallized from diethyl ether. 2-(2-Aminoethylamino)-6-(3-chlorophenylamino)-9-ethyl-9*H*-purine (130 mg, 49%) (CGP72693) is obtained; m.p. 79-80 °. ^{1}H-NMR (500 MHz, DMSO-d_6): d 1.37 (t, 3H, *J* = 7.5), 2.73 (t, 2H, *J* = 6.8), 3.30 (t, 2H, *J* = 6.8), 4.04 (q, 2H, *J* = 7.5), 6.71 (br, 1H), 6.98 (d, 1H, *J* = 9), 7.28 (t, 1H, *J* = 9), 7.89 (s, 1H), 7.93 (m, 1H), 8.28 (m, 1H), 9.60 (br, 1H). ^{13}C-NMR (90 MHz, DMSO-d6): d 16.2, 38.5, 42.3, 46.1, 114.8, 119.1, 120.1, 122.0, 130.8, 133.7, 139.0, 143.0, 152.7, 152.7, 160.0. Elemental analysis($C_{15}H_{18}N_7Cl$ .0.17 H_2O): calc. 53.80 H 5.52 N 29.28 Cl 10.59 H_2O 0.91; found C 53.90 H 5.62 N 28.86 Cl 10.80 H_2O 0.9. All described compounds are characterized by TLC, HPLC, ^{1}H-NMR, IR, mp if available. Most compounds are also characterized by elem. analysis (C,H,N) or HRMS.
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[32] Human bladder carcinoma T24 cells lines obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured according the protocol of the manufacturer, passaged twice a week and used from passages 54-74. Cells were seeded at 1.5 x 103 cells/well into 96-well microtiter plates and incubated overnight (16-18h) to allow cells to attach. Adherent cells were washed once with serum-free medium and exposed to serial dilutions of the drugs for 4-5 days. This allowed untreated control cultures to undergo at least 2-3 cell divisions. Individual drug concentrations were assayed in duplicates. After incubation, the cells were fixed with 3.3 % v/v glutaraldehyde, washed with water and stained with 0.05 % methylene blue. After washing, the dye was eluted with 3 % v/v HCl and the optical density measured at 665nm SpectraMax 340 (Bucher, Basel, Switzerland). IC50's were determined by a computerized system (SoftPro) using the formula (OD test - OD start) / (OD control - OD start) x 100. IC50 was defined as the drug concentration which leads to 50 % of cells per well compared to control cultures (100%) at the end of incubation period.
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