Synthesis and Biological Evaluation of N2-Substituted 2,4-Diamino-6-cyclohexylmethoxy-5-nitrosopyrimidines and Related 5-Cyano-NNO-azoxy Derivatives as Cyclin-Dependent Kinase2 (CDK2) Inhibitors

Synthesis and biological evaluation of new N2-substituted 2,4-diamino-6-cyclohexylmethoxy-5-nitrosopyrimidines and related 5-cyano-NNO-azoxy derivatives as CDK2 Inhibitors.

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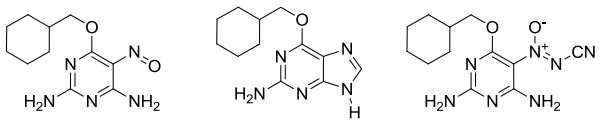
Abstract: The potent and selective Cyclin-Dependent Kinase 2 (CDK2) inhibitor NU6027 (6-cyclohexylmethoxy-5-nitroso-2,4-diaminopyrimidine) was used as the lead for the synthesis of a series of analogues in order to provide further insights into the structure activity relationships for 2,4-diaminopyrimidine CDK2 inhibitors. Aliphatic amino substituents were introduced at position 2. The use of linear or less sterically hindered amines gave rise to compounds endowed with slightly improved activity with respect to the lead; on the other hand, the compounds were the less active when a bulkier amino substituent was used. Substitution of the 5-nitroso group with the 5-cyano-*NNO*-azoxy moiety afforded a new class of inhibitors whose activity against CDK2 was similar to that of the nitroso series. The most active nitroso compound was **8b** (IC_{50} = 0.16 μM) while in the 5-cyano-*NNO*-azoxy series the most active compound was **9b** (IC_{50} = 0.30 μM). Taken as a whole these new analogues of **NU6027** enhance understanding of the structure activity relationships for 2,4-diaminopyrimidine CDK2 inhibitors.

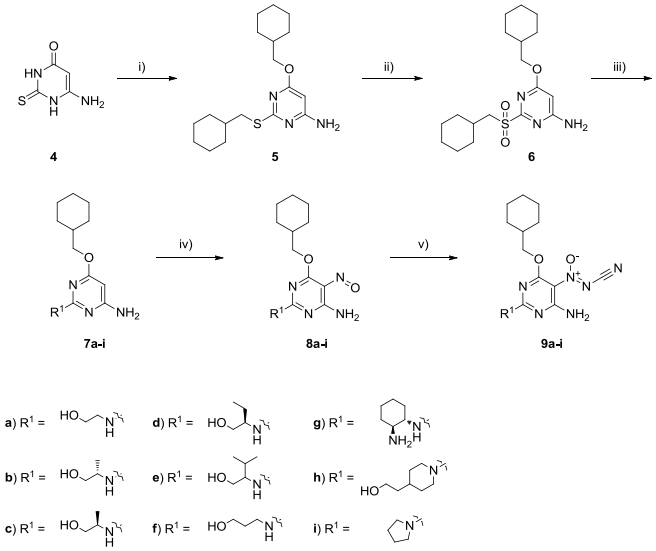
Cyclin-Dependent Kinases (CDKs) are serine/threonine kinases which display an abnormal activity in many kinds of tumours.[1] This family of kinases is represented by eleven members (CDK1-CDK11) and related cyclins.[2] Today, there is a great interest in small molecules able to behave as inhibitors of these enzymes as potential anticancer drugs. In the past decade, many such compounds belonging to different chemical classes have been developed.[3] Among these inhibitors, an interesting type is represented by 2,4-diamino-6-cyclohexylmethoxy-5-nitrosopyrimidine (**1**) (NU6027) (Chart 1), a competitive inhibitor of CDK1 and CDK2 isoforms with respect to ATP (CDK2 IC_{50} = 2.2 μM).[3-5] Owing to the intramolecular hydrogen bond between the adjacent 5-nitroso and 4-amino groups, this compound assumes a pseudo-purine geometry, which is reminiscent of the structure of 6-(cyclohexylmethoxy)-9*H*-purine (**2**) (NU2058) (Chart 1), an early relatively potent CDK1 and CDK2 inhibitor (CDK2 K_i = 12 μM).[6] Compound **1** can interact with the ATP-binding site of the enzymes by a triplet of hydrogen bonds (for CDK2: 2-NH_2 to Leu-83 (CO), N^3 to Leu-83 (NH), 4-NH_2 to Glu-81 (CO)).[4] These interactions exactly reproduce those of **2**. An extended series of analogues of **1** modified at the 2-,5- and 6- position(s) were synthesized in order to shed light on the structure-activity relationships (SAR) in this lead compound.[3, 4] In a recent paper we described a new pyrimidine scaffold, the 2,4-diamino-5-(cyano-*NNO*-azoxy)-6-(cyclohexylmethoxy)pyrimidine (**3**) (Chart 1), endowed with potent inhibitory CDK2 activity.[7] This substance can be formally obtained by substitution of the nitroso group of **1** with the , which is present in the antibiotic "calvatic acid" (4-[(*Z*)-cyano-*NNO*-azoxy]benzoic acid) isolated for the first time from cultural broth of *Calvatia lilacina.*[8]. This unusual functional group has been used to design several bioactive compounds, such as antimicrobial and antitumor agents, enzyme inhibitors, and calcium channel blockers.[9-13] The cyano-*NNO*-azoxy moiety displays an electron withdrawing property very similar to that of the nitroso group ($\sigma_{p\ NO}$ = 0.91; $\sigma_{p\ ONNCN}$ = 0.89), whereas it is endowed with different lipophilicity (π_{NO} = -1.20; π_{ONNCN} = -0.26) and steric properties.[14,15]



1 (NU6027) **2** (NU2058) **3**

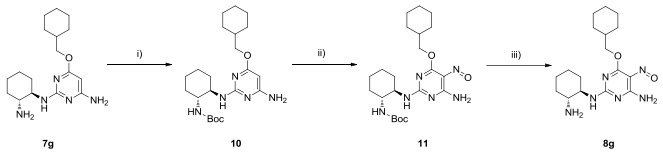
Chart 1. *Reference compounds.*

A molecular modelling study has suggested a role for a conserved water molecule in stabilizing the bioactive pose of 3 in the interaction with the ATP site of the enzyme. Preliminary SAR showed that the substitution of the cyano group of 3 with other electron withdrawing moieties induced a significant decrease in activity, whilst the introduction at the 2-NH2 group of a p-methylaminosulfonyl-substituted phenyl ring gave rise to a product whose potency fell in the nM range.[7] In this study a detailed description of the synthetic routes to a series of new N2-substituted examples of this structural class (**9a-i**) and of their 5-nitroso precursors (**8a-i**) is reported and the influence of the lateral chain at this position on the their CDK2 inhibitory activity is discussed.



Scheme 1. *Reagents and conditions: i) (bromomethyl)cyclohexane, 140°C μW heating, 16 min; ii) mCPBA, CH_2Cl_2, RT, 18 h; iii) R^1NH_2, 120°C, μW heating; iv) R^2ONO, DMSO, RT; v) NH_2CN, (diacetoxyiodo)benzene (IBA), CH_3CN, RT, 2 h.*

A series of N2-substituted 2,4-diamino-6-cyclohexylmethoxy-5-nitrosopyrimidines (**8a-i**) was prepared using a synthetic strategy (Scheme 1) similar to that described previously by Marchetti et al.[5] Some modifications improved the reported reaction conditions. Alkylation of 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**4**) was performed using (bromomethyl)cyclohexane. At variance with the previous synthetic strategy, (bromomethyl)cyclohexane was used to enable the concomitant alkylation at 2- and 4- positions. In addition, the use of microwave heating (μW) afforded the desired product 5 in 16 min at 140°C. Oxidation of 5 with mCPBA gave the corresponding cyclohexylmethylsulfone **6**. The nucleophilic displacement of cyclohexylsulfonyl group in 6 with diverse aliphatic amines was performed in an organic solvent (diglyme or THF) using microwave heating and in some cases, a Lewis acid such as $Yb(OSO_2CF_3)_3$ was added to improve the yield. The N2-substituted 4-amino-6-cyclohexylmethoxypyrimidines (**7a-i**) were thus obtained in moderate yield. Subsequent nitrosation at the 5-position with alkyl nitrites (menthyl nitrite or amyl nitrite), gave the desired nitroso compounds **8a-i**. The 5-nitroso derivative **8g** was obtained after $(Boc)_2O$ protection of compound **7g**, followed by nitrosation and subsequent deprotection of the amino group (Scheme 2). The final products (**9a-i**) were prepared by treating the nitroso derivatives (**8a-i**) with (diacetoxyiodo)benzene (IBA) and cyanamide (NH_2CN) in dry CH_3CN.[16]



Scheme 2. *Reagents and conditions: i) $(Boc)_2O$, THF, RT, 2 h; ii) menthyl nitrite, DMSO, RT, 18 h; iii) TFA, CH_2Cl_2, RT, 2 h.*

In the ^{1}H NMR experiments reported for the 5-nitroso derivatives (**8a-i**), the resonance of the 4-NH_2 group was split into two neat signals, which were observed at around 8 and 10 ppm, respectively. This observation was already reported in the previous work[7] and confirms the formation of an intramolecular hydrogen bond between the nitroso oxygen atom and one hydrogen of NH_2 group in the 4-position. In contrast, for the cyano-*NNO*-azoxy derivatives (**9a-i**), the signal of the 4-NH_2 group was reported as a broad singlet. In addition, ^{1}H and ^{13}C NMR spectra of both nitroso and cyano-*NNO*-azoxy derivatives presented two sets of resonances. This can be ascribed to the significant π component of the C2-N bond, as a consequence of strong conjugation between the electron donating N groups (e.g. $NHCH_3$ σp = -0.84)[14] and the strong electron withdrawing groups, the cyano-*NNO*-azoxy and the nitroso group respectively, with consequent restricted rotation around the C2-N bond at room temperature (**Figure 1**). This hypothesis was confirmed by a variable temperature NMR experiment (VT-NMR) performed on compound **8a** for which coalescence of the two sets of signals was observed at 125 °C.

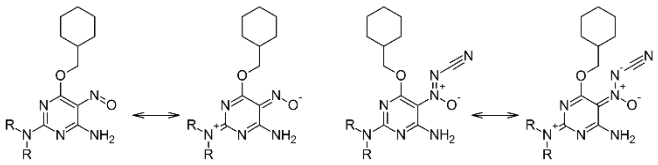
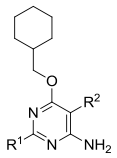
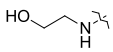
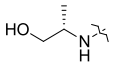
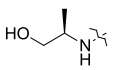
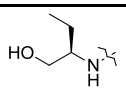
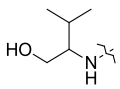
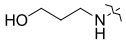
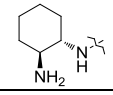
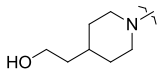


Figure 1. *Electronic conjugation between the electron releasing N group and the electron withdrawing NO or cyanoNNO-azoxy groups.*

Table 1. *Inhibitory activity of target compounds against CDK2.*



Compound	R^1	R^2	IC_{50} (μM)[a], [b]
1	NH_2	NO	2.2
3		-N(O)=N-CN	0.94
8a		NO	0.61
9a		-N(O)=N-CN	0.45
8b		NO	0.16
9b		-N(O)=N-CN	0.30
8c		NO	0.84
9c		-N(O)=N-CN	0.55
8d		NO	4.02
9d		-N(O)=N-CN	2.19
8e		NO	24.5
9e		-N(O)=N-CN	12.9
8f		NO	0.59

9f		-N(O)=N-CN	0.42
8g		NO	35% at 100 μM
9g		-N(O)=N-CN	66
8h			9% at 100 μM
9h			65.5
8i			49% at 100 μM
9i			54% at 100 μM

[a] All IC_{50} or % inhibition values are results obtained from n = 2 determinations.
[b] CDK assay conducted at 12.5 μM of ATP – full details in in reference.[17]

The new series of the 5-(cyano-*NNO*-azoxy) substituted compounds and of the related 5-nitroso precursors were evaluated for their CDK2 inhibitory activity using published procedures.[18] The results, expressed as IC_{50}, are shown in Table 1 together with the inhibitory potency of compounds **1** and **3** as references. The potency in the 5-nitroso series exhibits the order **8b**>**8f**≥**8a**>**8c**>**1**>**8d**>**8e**>**8i**≥**8g**>**8h**. Analysis of the data shows that the introduction of the hydroxyethyl group at the 2-NH_2 of **1** (**8a**) increases the inhibitory potency by about three-fold, whilst the introduction of the hydroxypropyl substructure gives rise to **8f**, which is about four-fold more potent than the reference. Also, the presence of the 2-hydroxy-1-methylethyl moiety (as in **8b**, **8c**) induces a potency increase that is particularly evident in the S-stereoisomer (**8b**). As for compounds **8d**,**e**,**g**, the activity decreases with the growing of the substituent in the 2-position. This is probably due to the weakening for steric reasons of the hydrogen bond between the 2-NH group of the compounds and the CO of the Leu-83 residue in the enzyme and to the fact that, due to the partially hindered rotation around C2-NH bond, one of the two conformers is unable to give this interaction. As expected, compounds **8h**,**i** displayed very feeble activity due to the absence of NH. The potencies and the SAR in the 5-(cyano-*NNO*-azoxy) series (**9a-i**) closely paralleled what was found in 5-nitroso series. Again, the most active substance **9b** bears the 2-hydroxy-1-methylethyl moiety at the 2-position and is about three-fold more active than the reference compound **3**.

The study reported in this paper extends the findings of the previous investigation.[7] A series of N2-substituted derivatives of the reference compound **1** was synthesized in order to explore structure-activity relationships concerning the substitution at this nitrogen position with aliphatic amino substituents present in relevant CDK inhibitors. Even though no significant improvements were achieved in term of biological activity, the SARs and understanding of the criteria for achieving CDK2 inhibitory activity were enhanced. Introduction of the 5-(cyano-*NNO*-azoxy) function gave no significant improvement comparing to the corresponding 5-nitroso derivatives. However, the cyano-*NNO*-azoxy group is a suitable replacement for the nitroso group at 5-position, able to maintain CDK2 inhibitory activity. Since these two moieties are endowed with different chemical and physical-chemical properties, they should give rise to two different classes of inhibitors, which should display different ADMET profiles worthy of additional investigation.

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Keywords: Cyclin Dependent Kinases (CDK), Anti tumor agents, CDK Inhibitors, Substituted pyrimidines, Nitrosation.

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