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Synthesis and In Vitro Evaluation of Novel 2,6,9-Trisubstituted Purines Acting as Cyclin-dependent Kinase Inhibitors

Michel Legraverend,[a] Odile Ludwig,[a] Emile Bisagni,[a] Sophie Leclerc,[b] Laurent Meijer,[b] Nicole Giocanti,[c] Ramin Sadri[c,†] and Vincent Favaudon[c,*]

[a]*UMR 176 CNRS-IC, Institut Curie-Recherche, Bât. 110-112, Centre Universitaire, 91405 Orsay Cedex, France*
[b]*Groupe Cycle Cellulaire, UPR 9042 CNRS, Station Biologique, BP 74, 29682 Roscoff Cedex, France*
[c]*Unité 350 INSERM, Institut Curie-Recherche, Bât. 110-112, Centre Universitaire, 91405 Orsay Cedex, France*

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Abstract—Novel C-2, C-6, N-9 trisubstituted purines derived from the olomoucine/roscovitine lead structure were synthesized and evaluated for their ability to inhibit starfish oocyte CDK1/cyclin B, neuronal CDK5/p35 and erk1 kinases in purified extracts. Structure–activity relationship studies showed that increased steric bulk at N-9 reduces the inhibitory potential whereas substitution of the aminoethanol C-2 side chain by various groups of different size (methyl, propyl, butyl, phenyl, benzyl) only slightly decreases the activity when compared to (*R*)-roscovitine. Optimal inhibitory activity against CDK5, CDK1 and CDK2, with IC_{50} values of 0.16, 0.45 and 0.65 μM, respectively, was obtained with compound **21** containing a (2*R*)-pyrrolidin-2-yl-methanol substituent at the C-2 and a 3-iodobenzylamino group at the C-6 of the purine. Compound **21** proved cytotoxic against human tumor HeLa cells ($LD_{50} = 6.7$ μM versus 42.7 μM for olomoucine, 24-h contact). Furthermore, unlike olomoucine, compound **21** was effective upon short exposure ($LD_{50} = 25.3$ μM, 2-h contact). The available data suggest that the affinity for CDKs and the cytotoxic potential of the drugs are inter-related. However, no straightforward cell cycle phase specificity of the cytotoxic response to **21** was observed in synchronized HeLa cells. With the noticeable exception of pronounced lengthening of the S-phase transit by **21** applied during early-S in synchronized HeLa cells, and in striking contrast with earlier reports on studies using plant or echinoderm cells, olomoucine and compound **21** were unable to reversibly arrest cell cycle progression in asynchronous growing HeLa cells. Some irreversible block in G1 and G2 phase occurred at high olomoucine concentration, correlated with induced cell death. Moreover, chronic exposure to lethal doses of compound **21** resulted in massive nuclear fragmentation, evocative of mitotic catastrophe with minor amounts of apoptosis only. It was also found that olomoucine and compound **21** reversibly block the intracellular uptake of nucleosides with high efficiency.

Introduction

Complexes of cyclins and cyclin-dependent kinases (CDKs) play a central role in the control of the cell division cycle.[1] The frequent deregulation of cell cycle progression in cancer[2] has prompted an active search for kinase inhibitors with high affinity and specificity for CDKs. Three major classes of CDK-targeting compounds have been identified to date, including butyrolactone I,[3,4] polyhydroxylated flavones such as flavopiridol,[5,6] and 2,6,9-trisubstituted purines.[7] The first substituted purine derivative with high CDK inhibitory activity, olomoucine (Scheme 1), was identified from screening against CDK1/cyclin B.[8] Among 35 kinases tested, olomoucine was found to competitively inhibit CDK1, CDK2, CDK5 and to a lesser extent erk1.[8] A more active 2,6,9-trisubstituted purine, roscovitine, has recently been described.[9–11]

The crystal structures of CDK2 complexes with olomoucine and (*R*)-roscovitine have been solved at 2.2–2.4 Å resolution.[9,12] Interestingly, the position of the purine nucleus in the ATP binding pocket of CDK2 differs markedly from that for the natural substrate, ATP. In addition, previous structure–activity relationship (SAR) studies[8,9] suggested that further structural variations at C-2, C-6 and N-9 might lead to more potent and more selective inhibitors of CDK2 and other CDKs. We have undertaken a new SAR program in order to test these possibilities and bring about novel, CDK-targeting trisubstituted purines acting below the micromolar

Key words: Purines; olomoucine; cyclin-dependent kinases; cell cycle; proliferation; human cells.

Abbreviations: BrdUrd, 5-bromo-2′-deoxyuridine. EGTA, ethylenebis(oxonitrilo)tetraacetate. SAR, Structure–activity relationship. EtOH, ethanol. CDK, cyclin-dependent kinase.

* Corresponding author. Tel.: 33-1-6986-3188; fax: 33-1-6986-3187; e-mail: vincent.favaudon@curie.u-psud.fr

† Present address: Department of Molecular and Chemical Endocrinology, St. Bartholomew's Hospital, 51–53 Bartholomew Close, London EC1A 7BE, UK.

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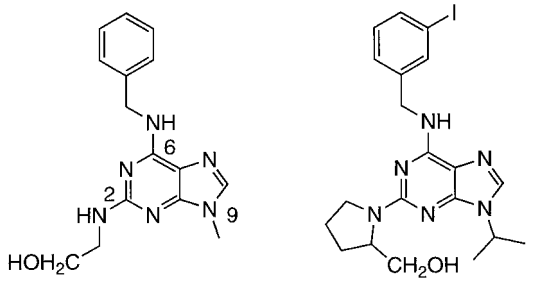


Olomoucine Compound **21**

Scheme 1. Chemical structure of olomoucine and compound **21**.

range, with the goal of providing new tools for cell cycle studies as well as pharmacological applications. Indeed, recent results point to both the antitumor potential of this family of purines,[13,14] and their ability to provide selective protection of neurons against apoptotic cell death.[15,16] It should also be stressed that CDK inhibitors might find utilization in non malignant disorders involving deregulated proliferation or metabolism such as psoriasis, restenosis,[17] arteriosclerosis, atherosclerosis, glomerulonephritis,[18] proliferation of unicellular parasites, cytomegalovirus infections[19] or Alzheimer's disease.[20] One compound in the series displayed sub-micromolar IC_{50}s against major CDKs. This prompted us to compare the effects of this new derivative to those of the parent compound olomoucine in a human cell line, including cytotoxicity studies, cell cycle phase specificity and redistribution, nucleoside import, and interaction with ionizing radiation. HeLa cells were chosen as a model system for this study, mostly because they do not express multi-drug resistance, grow as well-defined colonies and can be synchronized with great ease and efficiency. The results suggest that altered cell cycle progression and induced cell death by trisubstituted purines, may proceed through different pathways.

Results

General chemicals pathways

The preparation of 2,6-disubstituted purines has already been described using solid-phase[21] or solution-phase methodology.[22] The general synthetic pathway to trisubstituted purines used in the present work is outlined in Scheme 2 and employed 2,5-diamino-4,6-dichloropyrimidine **1**[23] as the starting material.* Selective C-4 monosubstitution of **1** was achieved by reaction with one equivalent of the appropriate amine in ethanol below 100 °C. Acid-catalyzed cyclization of **2** to **3** was carried out in *N*,*N*-dimethylacetamide at room temperature using an excess of triethylorthoformate. 6-Chloro-2-iodopurine (**4**) was subsequently obtained by diazotization-substitution of the 2 amino-6-chloropurine derivatives **3** using isoamylnitrite and a mixture of CH_2I_2, I_2 and CuI as the iodide source[25,26] in either THF or acetonitrile as solvent. When applied to cyclopentenyl precursor **3f**, this reaction led to a complex mixture from which **4f** could not be isolated. Nucleophilic substitution of the 6-chloro substituent was then specifically achieved by reaction with the amine below 45 °C. Further substitution of the 2-iodo function by another amine, required higher temperature (120–140 °C).

The structures of the compounds obtained in this way are described in Table 1. As clearly shown by the various examples described in this work, the synthetic strategy developed in this study allows unlimited variations at the 2, 6 and 9 positions of purine.

Structure–activity relationships

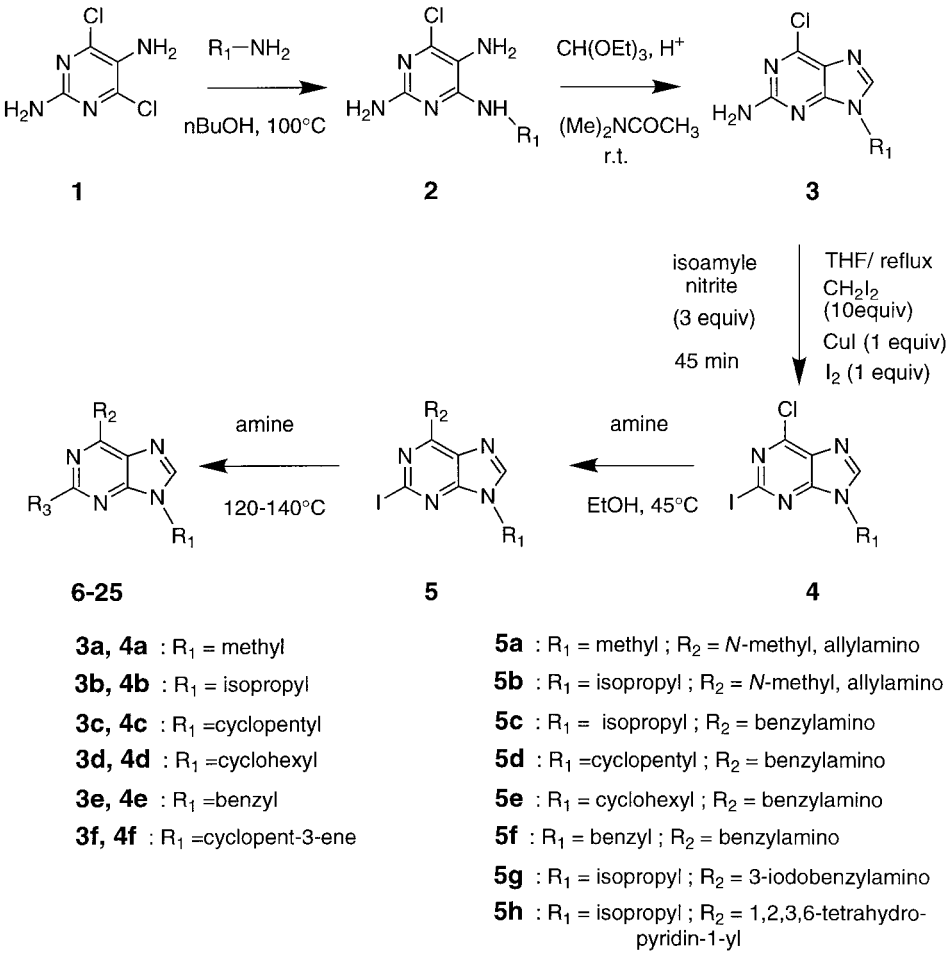
Previous preliminary structure–activity studies[8] of the purine ring have established that the presence of C-2, N-6 and N-9 substituents on adenine are critical for CDK1 inhibitory properties. These studies allowed identification of olomoucine[8] and subsequently of (*R*)-roscovitine[9,10] as new lead compounds that specifically inhibit CDK1, CDK2, CDK5 and, to a lesser extent, erk1.

The crystal structure of the CDK2-olomoucine complex at 2.2 Å resolution[12] and the CDK2-(*R*)-roscovitine complex at 2.4 Å resolution,[9] revealed 16 and 20 van der Waals interactions, respectively, between the drug N-6 benzyl substituent and the enzyme. We observed that replacement of the N-6 benzyl group by either an *N*-methyl allylamino group (**7**), or a 1,2,3,6-tetrahydropyridinyl system (**8**) strongly decreased inhibitory activity. However, increased anti-CDK1 and anti-CDK5 activities of the C-6-(3-iodobenzylamino) derivative **21** as compared to **10** should be noticed (Table 1).

Although an increased inhibitory effect against CDK1 was observed upon replacement of the 9-methyl substituent of olomoucine by an isopropyl group, as in (*R*)-roscovitine,[9] we found that, with the exception of the cyclopentyl derivative **24** which exhibited the same inhibitory activity as the isopropyl derivative **10**, a further increase in steric bulk and/or hydrophobicity at N-9 strongly decreases the inhibitory activity (Table 3). The C-2 side chain appears also to be essential in two ways. First, a hydrogen bond was detected between CDK2 Gln-131 and the hydroxyl group in olomoucine and (*R*)-roscovitine CDK2 co-crystals.[9,12] Second, substitution of the aminoethanol C-2 side chain by hydrophobic groups of varying size (methyl, propyl, butyl, phenyl, benzyl) slightly decreased the activity as compared to (*R*)-roscovitine (Table 1).

However, it is interesting to note the difference in activity between **9** and **10** in which the C-2 side chain is a 2-(*S*)-pyrrolidine methanol and 2-(*R*)-pyrrolidine methanol, respectively. Indeed, the 2-(*R*)-pyrrolidine methanol derivative **10** exhibits more potent inhibitory activity ($IC_{50} = 0.65\,\mu M$) than the (*S*) derivative **9** ($IC_{50} = 2.1\,\mu M$). The *R* configuration compound was always the

* The synthesis of some potent inhibitors of CDK2, using solid support technology, has been reported during the reviewing of this paper.[24]



Scheme 2. General pathways for the synthesis of the 2,6,9-trisubstituted purines in the series reported herein.

most active (Table 1), (i.e. **15** > **14**; **17** > **16**; **19** > **20**; **10** > **9**; and (*R*)-roscovitine (**28**) > (*R,S*)-roscovitine (**27**)).

The most active compound in this series was the 6-(3-iodobenzyl)-amino derivative **21** with an IC_{50} of 0.45, 0.65 and 0.16 μM against CDK1, CDK2 and CDK5, respectively. In fact, **21** was much more active than the lead compound olomoucine and as potent as (*R*)-roscovitine. As observed previously for olomoucine and (*R*)-roscovitine, all the CDK1 and CDK5 inhibitors (**9**–**24**) described in this paper were very weak or inactive against erk1 MAP kinase (Table 1). The high activity of **21** suggests that adjunction of bulkier lipophilic groups to the 6 benzylamino substituent may lead to other more potent compounds. Further experiments will be carried out to investigate this possibility.

Kinase effects

All compounds in the series were routinely probed for their ability to inhibit the CDK1/cyclin B complex from starfish oocytes. Due to the putative importance in Alzheimer disease of hyperphosphorylation of the microtubule-associated protein tau[27] and neurofilament subunits[28] by neuronal CDK5/p35, all compounds were also assayed on recombinant CDK5/p35. Thirdly, because olomoucine and (*R*)-roscovitine inhibit erk1 and erk2 at micromolar concentrations (IC_{50}s of 50 and 40 μM [olomoucine], 34 and 14 μM [(*R*)-roscovitine], for erk1 and erk2, respectively), we tested all compounds on purified recombinant erk1. The results are summarized in Table 1.

Compound **21** emerged as the most potent inhibitor in the series. It proved as efficient as (*R*)-roscovitine against CDK5, and was still more active against CDK1. However, in agreement with earlier results,[8] the data show that the inhibitory efficiencies against CDK1 and CDK5 are inter-related. With the exception of compound **22** (17 times more potent inhibitor of CDK5) and compound **15** (6 times more potent inhibitor of CDK1), no CDK5- or CDK1-specific inhibitor could be identified, confirming the close evolutionary relationship between the two kinases. On the other hand, most compounds at 10 μM concentration marginally inactivated erk1 kinase only. We also determined recombinant CDK2/cyclin E inhibition by olomoucine, (*R*)-roscovitine and compound **21**. Compound **21** again proved over 10-fold more active than olomoucine, and was as efficient as (*R*)-roscovitine in this assay (Table 2).

Table 1. Compared IC_{50}s of purine analogues in the olomoucine series for inhibition of purified erk1 kinase and p35/CDK5 or cyclinB/CDK1 complexes. Trivial names: compound **26**, olomoucine; compound **27**, (*R,S*)-roscovitine; compound **28**, (*R*)-roscovitine

Compound[a]	R_1	R_3	R_2	IC_{50} CDK1 (μM)	IC_{50} CDK5 (μM)	erk1 activity (%)[b]
3f	Cyclopent-3-ene	—	—	260	>100	97
4b	Isopropyl	—	—	150	>100	97
5a	Methyl	—	*N*-Methyl, allylamino	18	40	94
5b	Isopropyl	—	*N*-Methyl, allylamino	11	14	97
5c	Isopropyl	—	Benzylamino	8.0	13	88
5d	Cyclopentyl	—	Benzylamino	75	>100	94
5e	Cyclohexyl	—	Benzylamino	90	>100	91
5f	Benzyl	—	Benzylamino	100	>100	—
6	Methyl	2-Amino-ethanol	*N*-Methyl, allylamino	470	>100	97
7	Isopropyl	2-Amino-ethanol	*N*-Methyl, allylamino	150	>100	76
8	Isopropyl	2-Amino-ethanol	1,2,3,6-Tetrahydropyridin-1-yl	110	>100	100
9	Isopropyl	(2*S*)-Pyrrolidin-2-yl-methanol	Benzylamino	2.1	2.4	70
10	Isopropyl	(2*R*)-Pyrrolidin-2-yl-methanol	Benzylamino	0.65	1.3	69
11	Isopropyl	2-Benzyl-amino-ethanol	Benzylamino	2.5	8.0	94
12	Isopropyl	2-Amino-1-phenyl-ethanol	Benzylamino	6.5	2.3	88
13	Isopropyl	(2*R,S*)-2-Amino-hexanol	Benzylamino	2.5	2.3	93
14	Isopropyl	(2*S*)-2-Amino-2-phenyl-ethanol	Benzylamino	4.3	3.5	84
15	Isopropyl	(2*R*)-2-Amino-2-phenyl-ethanol	Benzylamino	1.0	6.0	84
16	Isopropyl	(2*S*)-2-Amino-2-benzyl-ethanol	Benzylamino	7.3	2.0	95
17	Isopropyl	(2*R*)-2-Amino-2-benzyl-ethanol	Benzylamino	2.7	1.3	89
18	Isopropyl	(2*R,S*)-2-Amino-pentanol	Benzylamino	0.9	0.4	77
19	Isopropyl	(2*R*)-2-Amino-propanol	Benzylamino	0.85	0.7	78
20	Isopropyl	(2*S*)-2-Amino-propanol	Benzylamino	1.0	35	83
21	Isopropyl	(2*R*)-Pyrrolidin-2-yl-methanol	3-Iodobenzylamino	0.45	0.16	76
22	Isopropyl	(3*R,S*)-Pyrrolidin-3-ol	Benzylamino	42	2.4	69
23	Cyclohexyl	(2*R*)-Pyrrolidin-2-yl-methanol	Benzylamino	40	13	99
24	Cyclopentyl	(2*R*)-Pyrrolidin-2-yl-methanol	Benzylamino	0.7	0.5	97
25	Benzyl	(2*R*)-Pyrrolidin-2-yl-methanol	Benzylamino	200	>100	97
26	Methyl	2-Amino-ethanol	Benzylamino	7.0	3.0	76
27	Isopropyl	(2*R,S*)-2-Amino-butanol	Benzylamino	0.65	0.16	77
28	Isopropyl	(2*R*)-2-Amino-butanol	Benzylamino	0.45	—	—

[a] See Scheme 2 for R_1, R_2, and R_3.
[b] Percent residual activity of erk1 kinase in the presence of 10 μM of various compounds.

Table 2. Compared inhibitory concentrations of olomoucine, (*R*)-roscovitine and compound **21** for the inactivation of purified recombinant CDK2/cyclin E complex (IC_{50}), and for loss of the clonogenic potential of asynchronously growing human tumor HeLa cells (LD_{50})

Drug	IC_{50} (μM)	Duration of contact with cells	LD_{50} (μM)
Olomoucine	7.0	16 h	156
Olomoucine		24 h	42.7
(*R*)-Roscovitine	0.70	24 h	6.6
Compound **21**	0.65	2 h	25.3
Compound **21**		24 h	6.7

Table 3. Effect of the N-9 substituent on the IC_{50} value relative to inhibition of CDK1/cyclin B activity

	N-9 substituent	IC_{50} (μM)
10	Isopropyl	0.65
24	Cyclopentyl	0.70
23	Cyclohexyl	40
25	Benzyl	200

Cytotoxicity assays

The viability of proliferating cells following exposure to olomoucine or compound **21**, was assessed in clonogenic assays. Even at the highest usable concentration, close to the limit of solubility of the drug, olomoucine in short (2 h) exposure did not produce any significant level of cytotoxicity. Olomoucine response increased steadily with the duration of exposure for up to 24 h, but further increase in the length of contact with drug had a marginal effect only. The cytotoxic potential of **21** also reached a plateau after 24-h exposure. However, compound **21** proved to be much more potent than olomoucine, and was cytotoxic from the very beginning of contact with drug (Table 2). Whatever the duration of drug exposure, the reproductive ability of HeLa cells decreased following a purely exponential dose–effect relationship for both drugs (data not shown).

Cell cycle phase specificity of compound 21 cytotoxicity

Cell cycle phase-specificity of the cytotoxic response is commonly observed with antitumor drugs, and may act as a major determinant of drug outcome. In order to investigate this issue, synchronized HeLa cells were exposed for 1 h to 50 μM **21** at graded times following release from a thymidine block. There was no significant change of HeLa cells killing by **21** with progression through the cell cycle (Fig. 1).

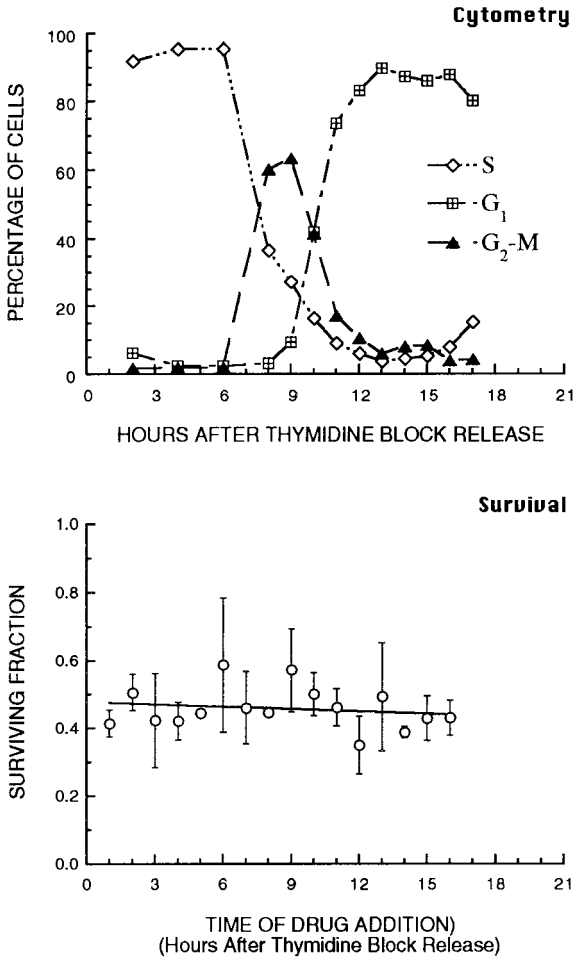


Figure 1. Cytotoxicity of compound **21** (50 μM, 1 h exposure) against HeLa cells progressing synchronously through the cell cycle following release from a double thymidine block. Flow cytometry (upper) and survival assays (lower) were carried out in parallel. Drugs were introduced at the times indicated. Survival data have been corrected for cell multiplicity.[54] Bars, SD.

Cell cycle phase redistribution by drugs

Asynchronous growing HeLa cells were exposed for extended periods of time to olomoucine or compound **21** at concentrations ranging from 1 to 100 μM, then collected after BrdUrd labeling, fixed in 70% EtOH and analyzed by flow cytometry to determine drug-induced cell cycle redistribution.

Incubation of HeLa cells with 100 μM olomoucine for 48 h resulted in pronounced S-phase depletion and minor accumulation of cells in the G2 phase, preceded by a transient block of cells in S-phase culminating at 24 h (Fig. 2). The G1 compartment evolved as the mirror image of S-phase, suggesting that the cell fraction exiting the G2 block through mitosis subsequently accumulated in G1. Limited effects only on cell cycle progression were observed at lower drug concentrations, with S-phase cells being the most reactive. Even at the highest olomoucine concentration (100 μM) and for the longest incubation time (48 h), no evidence for endoreduplication (polyploidy) or aberrant cytokinesis (multinucleation) was found. Moreover, the amount of hypodiploid nuclei remained to a basal level, less than 3% in terms of the DNA content as determined by flow cytometry, though less than 5% of the bulk cell population was able to resume growth after drug removal under these conditions.

The situation was substantially different with compound **21**. First of all, exposure to 25 μM compound **21** depressed the ability of cells to incorporate BrdUrd, consistent with inhibition of DNA synthesis in S-phase and/or of nucleoside uptake (see below). Second, flow cytometric analysis showed a high level of micronucleation yielding nuclei with random hypodiploid and hypotetraploid DNA content (Fig. 3). Agarose gel electrophoresis of DNA extracts did not get evidence of the DNA ladders characteristic of apoptosis (data not shown). The amount of cells undergoing nuclear fragmentation upon chronic exposure to 25 μM **21** was considerably higher than with 100 μM olomoucine, and reached completion upon 48-h incubation. This notwithstanding, fragmentation did not occur at and below 12.5 μM compound **21** (48-h incubation), yet less than 10% of cells survived this treatment. Otherwise, S-phase depletion was the main feature characterizing altered cell cycle progression by compound **21**. The potency of **21** to arrest cells in G2 seemed relatively minor compared to that for olomoucine (Fig. 2).

Altered S-phase progression by compound 21 in synchronized cells

Although cytotoxicity assays did not provide evidence for cell cycle phase specificity of HeLa cell killing, we reasoned that if it occurred, the use of synchronized HeLa cells could allow for a more precise determination of altered cell cycle progression by olomoucine derivatives. Synchronized HeLa cells were thus exposed briefly (1 h) to 50 μM **21** at different times following release from a thymidine block, and collected 24 h after block release for flow cytometric analysis. Cells in untreated samples were exactly halfway through the second S-phase following thymidine block release, having successively traversed S-, G2-, M-, and G1-phases. Pulse contact with **21** was found to slow down cell cycle traversal. The effect was more pronounced as the drug was given early in S-phase in the first cycle, and disappeared progressively thereafter. Indeed, a 1-h exposure to drug in early-S phase delayed entry into the next S-phase by as much as 5 h (Fig. 4).

Inhibition of thymidine uptake by drugs

Olomoucine and compound **21** were tested for their ability to alter uptake and incorporation of radioactively labeled thymidine by living cells, following a procedure similar to that described by Buquet-Fagot et al.[29] Both drugs actually inhibited thymidine import (Fig. 5). The process exactly fitted an equilibrium equation, with K_d values in the same range as those for CDKs in acellular extracts (Tables 1 and 2), and was fully reversible upon drug removal.

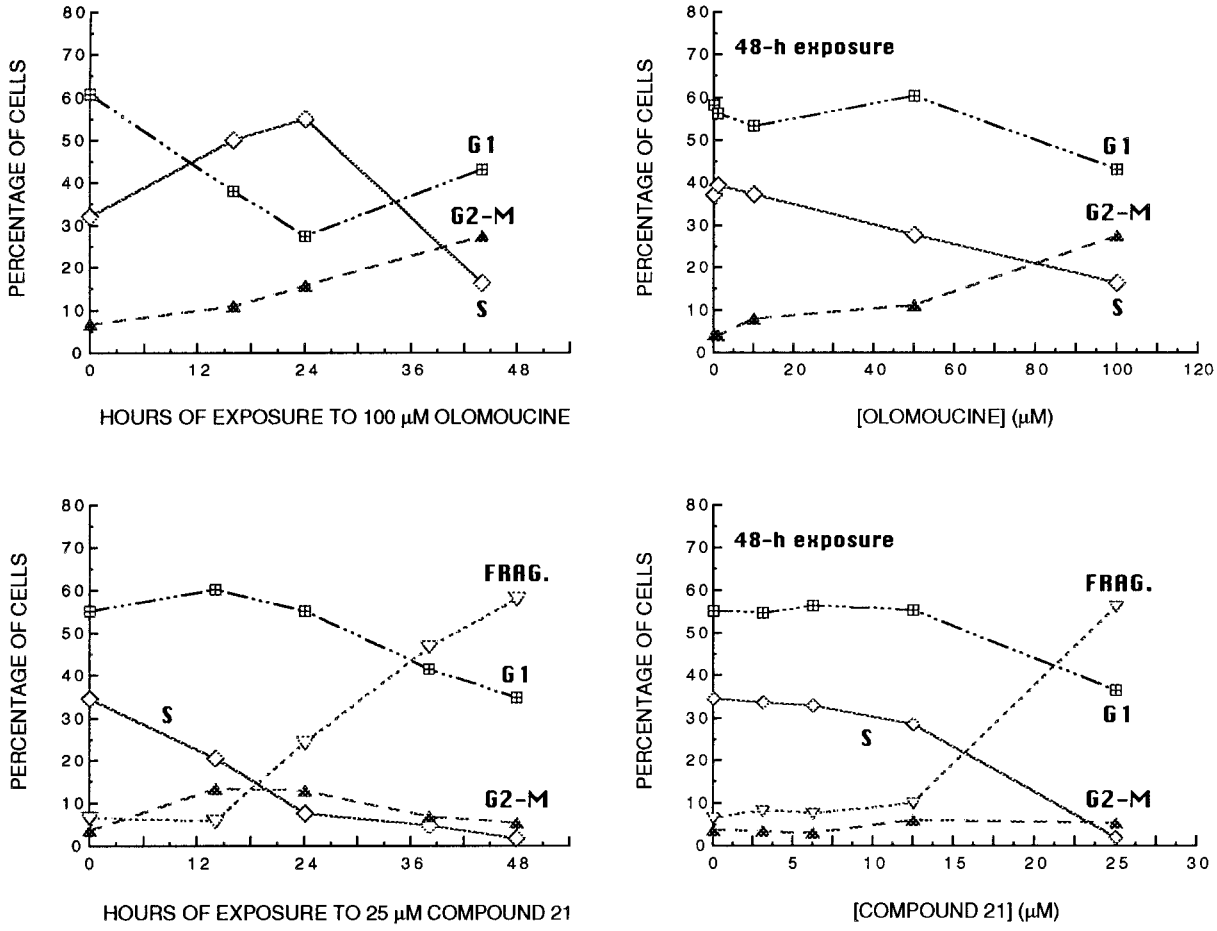


Figure 2. Time and drug concentration-dependence of cell cycle redistribution by olomoucine and compound **21** in asynchronously growing cultures of HeLa cells. The length of contact with drug in the drug concentration dependence assay was 48 h. FRAG refers to total hypodiploid and hypotetraploid nuclear fragments generated by the treatment and measured by cytofluorimetry in the same way as in Figure 3.

Altered radiation response by drugs

Near-suppression of the initial slope (α) of the radiation survival curve was observed as cells were exposed to compound **21** (Fig. 6). Survival curves in the high dose range with or without drug were parallel, and in fact the presence of drug did not change significantly the value of the quadratic (β) parameter characterizing high dose radiation survival. The same effect was observed with 100 μM olomoucine or 60 μM (*R*)-roscovitine (data not shown). Therefore, short exposure to cytotoxic concentrations of olomoucine derivatives applied concomitantly with radiation results in very efficient radiation protection selectively at low radiation doses, but does not hinder radiation-induced cell kill at high radiation doses.

Discussion

The series of purines described in this paper are close structural analogues of the CDK inhibitors olomoucine and (*R*)-roscovitine. Kinase assays for this new series confirm that C-2, C-6, and N-9 substituted purines are a rich source of selective CDK-targeting inhibitors. Note, however, that substitution of the N-9 position by bulky groups results in loss of the inhibitory potential. In contrast, both C-2 and C-6 positions allow a large variety of variations, leading to substantial changes in the inhibitory strength. Crystal structure analysis[9,12] has identified the benzyl ring as the modification responsible for the selectivity of this family of compounds towards CDKs; this motif interacts with amino acid residues which are not conserved among kinases, and which do not interact with ATP. This region of the molecule will be the focus of future work. The C-2 position allows a large variety of substitutions. Finally, optimal activity was observed with a (*R*)-pyrrolidine methanol substituent at the C-2 of the purine whereas, as previously observed,[8,11] an isopropyl substituent at N-9 gave the best activity. The high activity of **21** against CDK1, CDK2 and CDK5 corroborated previous observations[22,30] indicating that substitution of the 6-benzylamino substituent by bulky lipophilic groups may lead to more potent compounds. Our data show that human HeLa cell response to substituted purines differs markedly from what might be anticipated from studies on cell extracts or purified enzymes in the test tube, as well as from effects found in algae, plants, echinoderms cells or oocytes. In particular, olomoucine and/or (*R*)-roscovitine reportedly induce reversible arrest of plant cells in late-G1 and late-G2 phases of the cell cycle,[31,32] inhibit

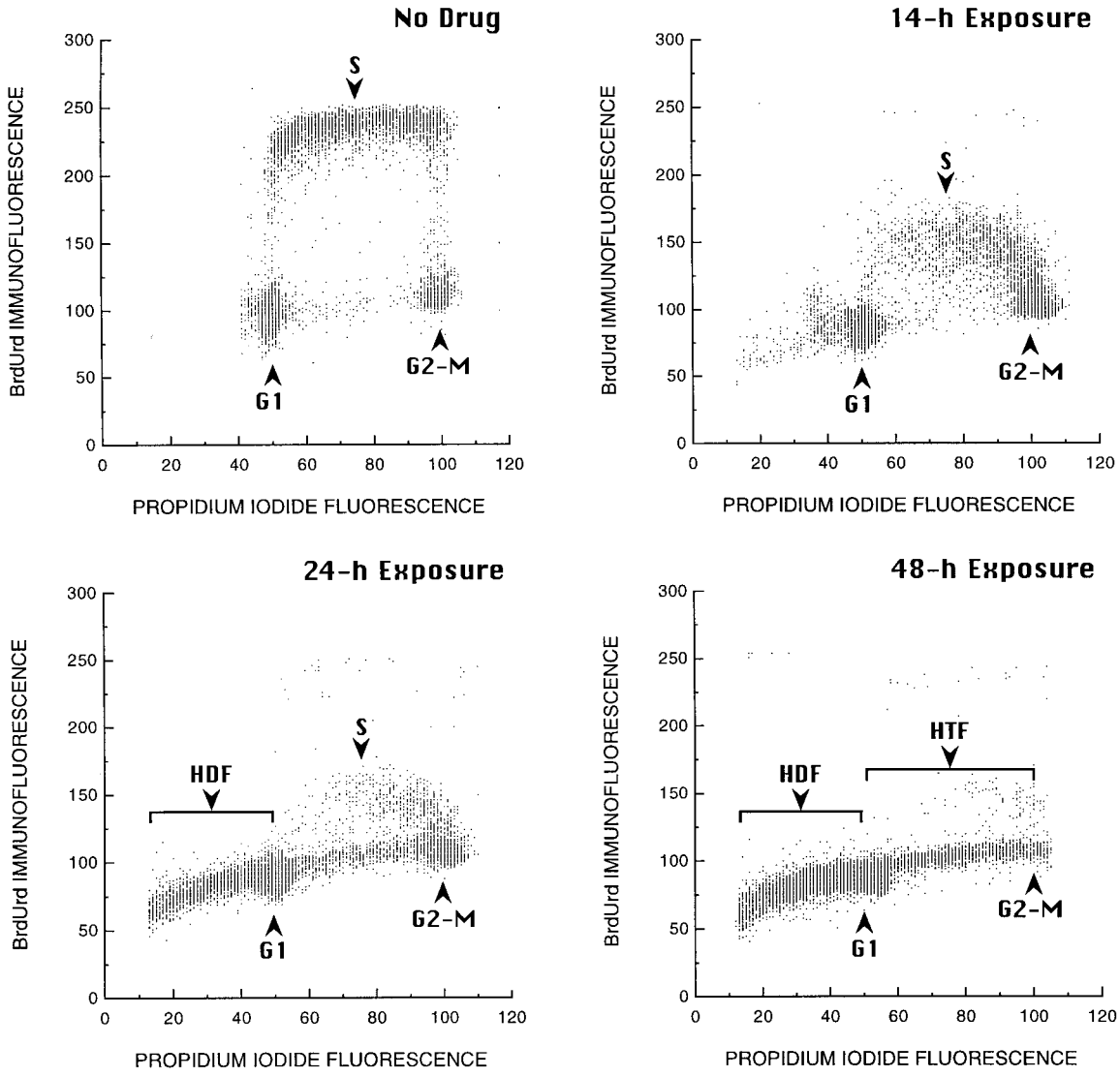


Figure 3. Dot plot cytograms showing cell cycle redistribution and micronucleation induced by compound **21** (25 μM) in HeLa cells as a function of the length of contact with drug. Data were obtained from bivariate cytofluorimetric analysis, based on concomitant DNA fluorescence (propidium iodide) and immunofluorescence (BrdUrd) detection (see Experimental). The propidium iodide signal (FL3) is proportional to the DNA content. The BrdUrd signal (FL1) was converted through an 8-bits (256 channels) logarithmic amplifier calibrated versus maximum fluorescence emission. Each dot represents a particular nucleus or nuclear fragment. HDF, hypodiploid fragments; HTF, hypotetraploid fragments.

the maturation of starfish oocytes,[10,33] and arrest the growth of Xenopus oocytes and sea urchin eggs.[10] Both purines thus have the ability to block cell cycle progression at definite checkpoints, suggesting that CDK2/cyclin A, CDK2/cyclin E and CDK1/cyclin B complexes are major targets for these drugs in these species. In keeping with these results, we found that olomoucine and compound **21** (the most active compound in the series) did not produce reversible accumulation of HeLa cells in G1 and G2 phase. When observed, such blocks were irreversible and correlated with large amounts of induced cell death. These results are in close agreement with those reported by Schutte et al.[34] and Buquet-Fagot et al.[29] Therefore, CDKs may not be the only critical targets for substituted purine-induced cell death in human cells. Brüsselbach et al.[35] working on the flavone derivative flavopiridol, another potent CDK inhibitor, recently reached similar conclusions. Furthermore, HeLa cells did not show any particular cell cycle phase-dependent cytotoxic response to the drug (Fig. 1). Differences in the efficiency of drug uptake might explain such differential response of various species or cell lines for induced killing or altered cell cycle regulation by substituted purines. Indeed, as demonstrated by Buquet-Fagot et al.[29] exposure of growing cells to olomoucine or (*R*)-roscovitine results in rapid, efficient inhibition of nucleoside intake. Figure 5 shows that this inhibitory process obeys an equilibrium, with K_d values in the same range as those determined for purified CDK1 (Table 1) or CDK2 (Table 2). This suggests that purine derivatives in the olomoucine series act as competitive inhibitors at the ATP binding site of a protein involved in the active transport of nucleosides, in rather the same way as for the ATP binding pocket of CDKs. This may produce self-inhibition of purine import, and contribute to drug-induced lethality upon prolonged

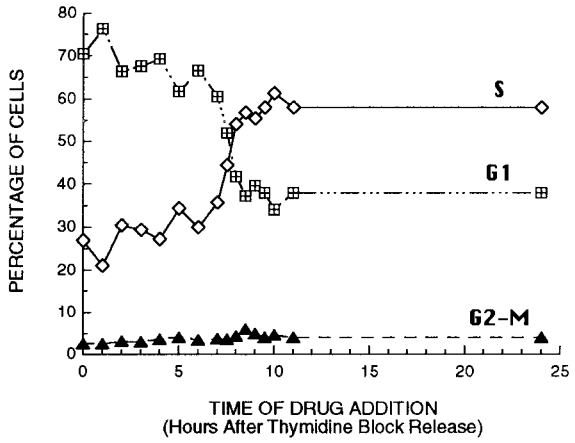


Figure 4. Characterization of the cell cycle delay produced by pulse exposure to compound **21**. HeLa cells were synchronized by a double thymidine block and exposed to **21** (50 μM, 1 h contact) at different times following block release. At 24 h after block release, cells were labeled with BrdUrd (10 μM, 15 min), trypsinized, fixed in 70% cold EtOH and subsequently examined by flow cytometry. Untreated cells at that time were exactly halfway through the second S-phase following synchronization. Delay to the second S-phase was the larger as **21** was given closer to the beginning of the first S-phase. Cell cycle progression in drug-free synchronized cells is shown in Figure 1.

drug exposure. Side effects from altered microtubule dynamics by impaired kinase activity[36] might also occur and contribute to the lethal effect of the olomoucine derivatives.

Nevertheless, there is a striking parallel between the affinity for CDKs and the cytotoxic potential of trisubstituted purines, and these appear to be able to target specific cell cycle compartments in living HeLa cells. In

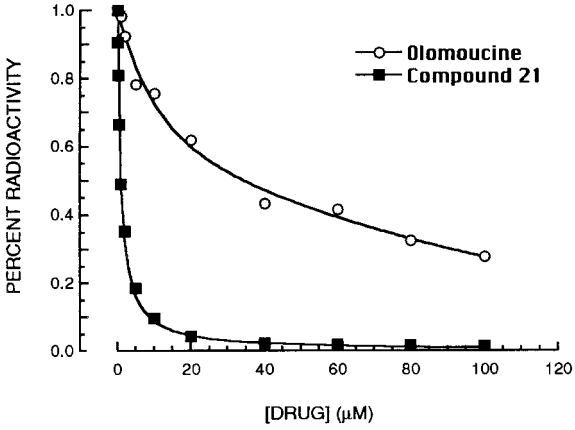


Figure 5. Inhibition of thymidine uptake by olomoucine or **21**. Asynchronous growing HeLa cells ($1.00 \pm 0.05 \times 10^6$ cells) were incubated with drugs for 90 min prior to 30 min contact with [methyl-^{3}H]thymidine (see Experimental). The process was analyzed as a reversible equilibrium,

$$E + D \rightleftarrows ED$$

where E is a putative membrane receptor involved in the active transport of thymidine, D the drug, and ED the enzyme–drug complex. With the most active compound, thymidine intake was abolished at high drug concentration, indicating that the ED complex is totally unable to carry out thymidine import. K_d values were calculated at 24.1 ± 1.0 μM for olomoucine and 0.948 ± 0.014 μM for compound **21**.

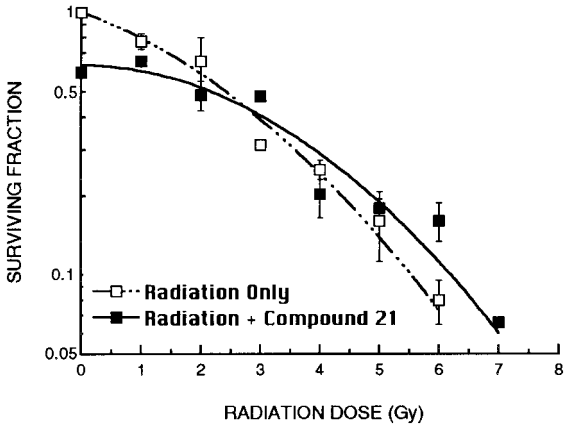


Figure 6. γ-Ray survival of asynchronous growing HeLa cells without or with concomitant treatment with compound **21**. Drug (40 μM) was introduced 1 h prior to irradiation and was present for a total of 2 h. Survival curves were fitted to the linear-quadratic eq (2) (see Experimental). Found, $\alpha = 0.186 \pm 0.054$ Gy^{-1} and $\beta = 0.0418 \pm 0.0016$ Gy^{-2} for radiation alone; $\alpha = 0.0030 \pm 0.0006$ Gy^{-1} and $\beta = 0.0475 \pm 0.0074$ Gy^{-2} in combined treatment with **21**; fractional survival to **21** alone was 0.63. Bars, SD.

particular, compound **21** induced a prolonged, yet reversible lag in progression through the S-phase of the cell cycle in synchronized HeLa cells. Susceptibility to this effect peaked in early-S, decreased with progression through S-phase, and disappeared as cells reached the S-G2 transition (Fig. 4). This gives circumstantial evidence in favor of CDK2/cyclin E and CDK2/cyclin A complexes being major targets of olomoucine analogues in HeLa cells. As a matter of fact, both cyclin E and cyclin A are considerably overexpressed in cells synchronized at the G1-S boundary by thymidine,[37] thus putatively offering increased substrate concentration for drug action. Moreover, early events in DNA replication require CDK2/cyclin E[38] and CDK2/cyclin A,[39,40] while CDK2/cyclin A is necessary to progression through S phase.[41] It is thus conceivable that inhibition of CDK2/cyclin E or CDK2/cyclin A activity may result in slowing down early-S-phase progression in some cell lines. Furthermore, it has long been known that S-phase is the most radioresistant compartment of the cell cycle.[42–45] Reducing the rate of the S-phase transit is expected to provide more time for radiation-induced DNA damage repair, thus resulting in substantial enhancement of the radiation resistance among drug survivors, consistently with the effect we observed in the low radiation dose range (Fig. 6).

Experimental

Chemistry

Analytical methods. The melting points were taken on a Kofler hot stage apparatus, and are uncorrected. Elemental analyses were performed by the Service de Microanalyse, CNRS, ICSN, Gif-sur-Yvette, France. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 200 MHz on a Bruker AC-200 spectrometer. Chemical shift values are reported in δ (ppm), relative to tetramethylsilane as an internal standard.

Preparation of compounds 3a–f. In separate experiments, methylamine, isopropylamine, cyclopentylamine, cyclohexylamine, benzylamine (purchased from Aldrich) or cyclopent-3-ene-1-yl-amine[46] were treated with 1.1 equiv of 2,5-diamino-4,6-dichloropyrimidine (**1**) in *n*-BuOH containing an excess of triethylamine at 100 °C for 48 h (argon atmosphere).[47] Compounds **2** were then cyclized to **3a–f** at room temperature by treatment with triethyl orthoformate in *N*,*N*-dimethylacetamide, containing a catalytic amount of aqueous concentrated HCl.

2-Amino-6-chloro-9-methyl-9*H*-purine (3a). Yield from **1** 78%; mp sublimate at 240 °C; ^{1}H NMR (DMSO-d_6), δ 8.07 (s, 1H), 6.90 (s, 2H), 3.63 (s, 3H). Anal. ($C_6H_6N_5Cl$) C,H,N.

2-Amino-6-chloro-9-isopropyl-9*H*-purine (3b). Yield from **1** 94%; mp (H_2O) 139 °C; ^{1}H NMR (DMSO-d_6), δ 8.24 (s, 1H), 6.88 (s, 2H), 4.61 (m, 1H), 1.49 (d, 6H, 2×CH_3, J = 6.7 Hz). Anal. ($C_8H_{10}N_5Cl$) C,H,N.

2-Amino-6-chloro-9-cyclopentyl-9*H*-purine (3c). Yield from **1** 91%; mp (H_2O) 142 °C; ^{1}H NMR (DMSO-d_6), δ 8.21 (s, 1H), 6.88 (s, 2H), 4.70 (m, 1H), 2.5–1.6 (m, 8H). Anal. ($C_{10}H_{12}N_5Cl$) C,H,N.

2-Amino-6-chloro-9-cyclohexyl-9*H*-purine (3d). Yield from **1** 80%; mp 165 °C; ^{1}H NMR (DMSO-d_6), δ 8.23 (s, 1H), 6.89 (s, 2H), 4.22 (m, 1H), 1.93–130 (m, 10 H). Anal. ($C_{11}H_{14}N_5Cl$) C,H,N.

2-Amino-6-chloro-9-benzyl-9*H*-purine (3e). Yield from **1** 82%; mp 210 °C; ^{1}H NMR (DMSO-d_6), δ 8.23 (s, 1H), 7.3 (m, 5H), 6.94 (s, 2H), 5.29 (s, 2H). Anal. ($C_{12}H_{10}N_5Cl$) C,H,N.

2-Amino-6-chloro-9-cyclopent-3-ene-1-yl-9*H*-purine (3f). Yield from **1** 82%; mp (ethanol) 149–150 °C; ^{1}H NMR (DMSO-d_6), δ 8.04 (s, 1H), 6.89 (s, 2H), 5.87 (s, 2H) 5.09 (m, 1H), 2.95–2.62 (m, 4H). Anal. ($C_{10}H_{10}N_5Cl$) C,H,N.

Preparation of compounds 4a–e. Preparation of compounds **4a–e** from **3a–e** was achieved as described by Matsuda et al.[26]

6-Chloro-2-iodo-9-methyl-9*H*-purine (4a). Yield 42%; mp (ethanol) 194–196 °C; ^{1}H NMR (DMSO-d_6), δ 8.04 (s, 1H), 6.89 (s, 2H), 5.87 (s, 2H) 5.09 (m, 1H), 2.95–2.62 (m, 4H). Anal. ($C_6H_4N_4ClI$) C,H,N.

6-Chloro-2-iodo-9-isopropyl-9*H*-purine (4b). Yield 82%; mp (heptane) 108–109 °C; ^{1}H NMR (DMSO-d_6), δ 8.76 (s, 1H), 4.83 (m, 1H), 1.55 (d, 6H, J = 6.7 Hz). Anal. ($C_8H_8N_4ClI$) C,H,N.

6-Chloro-2-iodo-9-cyclopentyl-9*H*-purine (4c). Yield 56%; mp (cyclohexane) 106–107 °C; ^{1}H NMR (DMSO-d_6), δ 8.73 (s, 1H), 4.92 (m, 1H), 2.52–1.71 (m, 8H). Anal. ($C_{10}H_{10}N_4ClI$) C,H,N.

6-Chloro-2-iodo-9-cyclohexyl-9*H*-purine (4d). Yield 55%; mp (cyclohexane) 133–134 °C; ^{1}H NMR (DMSO-d_6), δ 8.75 (s, 1H), 4.44 (m, 1H), 2.00–1.44 (m, 10H). Anal. ($C_{11}H_{12}N_4ClI$) C,H,N.

6-Chloro-2-iodo-9-benzyl-9*H*-purine (4e). Yield 55%; mp (heptane) 136–137 °C; ^{1}H NMR (DMSO-d_6), δ 8.73 (s, 1H), 7.30 (m, 5H), 5.48 (s, 2H). Anal. ($C_{12}H_8N_4ClI$) C,H,N.

Preparation of compounds 5a–h. Substitution of the 6-chloro in **4a–e** was routinely achieved by reaction with 1.1 equiv of the appropriate amine (*N*-methylallylamine, benzylamine, 3-iodobenzylamine or 1,2,3,4-tetrahydropyridine, purchased from Aldrich) in EtOH containing $N(Et)_3$ (4 equiv) under nitrogen at 45 °C for 3 h. The solvent was then removed in vacuo. The residue was dissolved in CH_2Cl_2 and washed twice with water. The organic phase was dried ($MgSO_4$), evaporated to dryness and crystallized. Flash chromatography purification on silica gel column eluting with 5% ethanol in CH_2Cl_2 was necessary in the case of **5b**, **5c** and **5f** (Table 4).

Preparation of compounds 6–25. Starting materials were purchased from Aldrich: ethanolamine, (*S*)-(+)-2-pyrrolidine methanol, (*R*)-(−)-2-pyrrolidine methanol, *N*-benzylethanolamine, 2-amino-1-phenylethanol, DL-2-amino-1-hexanol, (*S*)-(+)-2-phenylglycinol, (*R*)-(−)-2-phenylglycinol, (*S*)-(−)-2-amino-3-phenyl-1-propanol, (*R*)-(+)-2-amino-3-phenyl-1-propanol, DL-2-amino-1-pentanol, (*S*)-(+)-2-amino-1-propanol, (*R*)-(−)-2-amino-1-propanol, 3-pyrrolidinol.

In a typical procedure, a mixture of 2-iodo purine derivative **5c** (2 mmol) in *N*,*N*-dimethylacetamide (40 mL), tripropylamine (1 mL) and 1.5 equiv of (*R*)-(−)-2-pyrrolidine methanol was stirred at 140 °C for 24 h. In the case of **12–19** and **20**, the mixture was placed in a sealed tube filled with glass beads, and was heated at 140 °C. After evaporation to dryness (oil pump), the residue was dissolved in CH_2Cl_2, washed with water (twice) adsorbed on silica gel and chromatographed on a silica gel column eluting successively with CH_2Cl_2 and 2–5% EtOH in CH_2Cl_2. In general crystalline products were obtained (see Table 4). NMR data for compounds **5a–h** and **6–25** are presented in Table 5.

Biological studies

Kinase assays. Unless otherwise specified, kinase activities were assayed in buffer C (see below), at 30 °C, in the presence of 15 μM ATP. Blank values were subtracted from the data and activities were calculated as pmoles of phosphate incorporated in protein acceptor after 10 min incubation. Controls were performed with appropriate dilutions of DMSO. In a few cases phosphorylation of the substrate was assessed by autoradiography after polyacrylamide gel electrophoresis in the presence of lauryl sulfate.

CDK1/cyclin B was extracted in homogenization buffer from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on $p9^{CKShs1}$-sepharose beads, from which it was eluted by free $p9^{CKShs1}$ as previously described.[10,48] The kinase activity

Table 4. Physico-chemical data for selected compounds

Compound	Mp (°C)	% Yield	Formula	Analysis	Recrystallization solvent
5a	122–123	98	$C_{10}H_{12}N_5I$	C,H,N	Cyclohexane
5b	Oil	86	$C_{12}H_{16}N_5I$	C,H,N	—
5c	182	70	$C_{15}H_{16}N_5I$	C,H,N	Cyclohexane
5d	213–214	90	$C_{17}H_{18}N_5I$	C,H,N	Dichloromethane
5e	210–212	88	$C_{18}H_{20}N_5I$	C,H,N	Dichloromethane
5f	184–185	77	$C_{19}H_{16}N_5I$	C,H,N	Dichloromethane
5g	176–178	93	$C_{15}H_{15}N_5I_2$	C,H,N,I	EtOH
5h	94–98	98	$C_{13}H_{16}N_5I$	C,H,N	EtOH/H_2O
6	Oil	57	$C_{12}H_{18}N_6O$	C,H,N	—
7	Oil	91	$C_{14}H_{22}N_6O$, 1/2H_2O	C,H,N	—
8	Oil	69	$C_{15}H_{22}N_6O$	C,H,N	—
9	116–117		$C_{20}H_{26}N_6O$	C,H,N	Diethyl ether
10	Oil	62	$C_{20}H_{26}N_6O$	C,H,N	—
11	124–126	52	$C_{24}H_{28}N_6O$	C,H,N	Cyclohexane
12	172–174	85	$C_{23}H_{26}N_6O$	C,H,N	Dichloromethane
13	149–151	58	$C_{21}H_{30}N_6O$	C,H,N	Dichloromethane
14	132–134	47	$C_{23}H_{26}N_6O$, 1/2H_2O	C,H,N	EtOH/H_2O
15	130–131	90	$C_{23}H_{26}N_6O$, 1/2H_2O	C,H,N	EtOH/H_2O
16	148–150	73	$C_{24}H_{28}N_6O$	C,H,N	EtOH/H_2O
17	150–151	60	$C_{24}H_{28}N_6O$	C,H,N	Cyclohexane
18	172–174	55	$C_{20}H_{28}N_6O$	C,H,N	Dichloromethane
19	112–114	43	$C_{18}H_{24}N_6O$	C,H,N	Cyclohexane
20	112–113	47	$C_{18}H_{24}N_6O$	C,H,N	Cyclohexane
21	152–154	84	$C_{20}H_{25}N_6OI$	C,H,N,I	EtOH
22	167–168	37	$C_{19}H_{24}N_6O$	C,H,N	Cyclohexane
23	151–152	50	$C_{23}H_{30}N_6O$	C,H,N	Diethyl ether
24	147–149	87	$C_{22}H_{28}N_6O$	C,H,N	Diethyl ether
25	129–131	89	$C_{24}H_{26}N_6O$	C,H,N	Diethyl ether

[a] Yields refer to isolated and purified materials.
[b] All compounds were analyzed within ±0.4% of the theoretical values.

was assayed with 1 mg/mL histone H1 (Sigma type III-S), in the presence of 15 μM [γ-^{32}P]ATP (3,000 Ci/mmol; 1 mCi/mL) in a final volume of 30 μL. After 10 min incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto 2.5×3 cm pieces of Whatman P81 phosphocellulose paper, and, after 20 s, the filters were washed five times (for at least 5 min each time) in a solution of 10 mL phosphoric acid/liter of water. The wet filters were transferred into 6 mL plastic scintillation vials, 5 mL ACS (Amersham) scintillation fluid was added and the radioactivity measured in a Packard counter. The kinase activity was expressed in pmoles phosphate incorporated in histone H1 per 10 min incubation, or in % of maximal activity. CDK2/cyclin E (provided by Dr. W. Harper) and His-tagged erk1 (provided by Dr. M. Cobb) were assayed as described.[10]

Homogenization buffer was made of 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl-phosphate, 25 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor and 100 μM benzamidine. Buffer C was identical to the homogenization buffer but did not contain EGTA, NaF and protease inhibitors.

Reagents for biological investigations. Thymidine, BrdUrd and propidium iodide came from Sigma. Distilled DMSO (spectroscopy grade) was purchased from Merck. All products for cell culture were from Life Technologies/Gibco-BRL. [Methyl-^{3}H]thymidine was obtained from New England Nuclear Life Science Products. Rat monoclonal antibody directed against BrdUrd was purchased from CeraLab. Fluorescein isothiocyanate-conjugated goat anti-rat IgG (heavy chain specific) was purchased from Southern Biotechnology Associates.

Cell cultures. Human HeLa (cervix carcinoma) cells were kindly provided by Dr. J. Coppey (Institut Curie). Cells were checked to be free of mycoplasma contamination, and routinely subcultured in 25 cm^2 flasks (Becton–Dickinson) every 4 days at a density of 1–2×10^5 cells/flask in Dulbecco's modified Eagle's minimal essential medium with 4.5 g/L glucose, 0.1 g/L pyruvate, 10^5 UI/L penicilline, 0.1 g/L streptomycine, 0.86 g/L Glutamax I and 10% v/v fetal calf serum (37 °C, 7% CO_2). The number of passages from the same primary subculture was kept below 7–8. The mean doubling time (mid-log phase) was 21 ± 1 h. Control plating efficiencies were in the range of 70%.

Synchronization of HeLa cell cultures at the G1-S junction was performed using a double thymidine block technique as described.[49] The method does not affect the viability of cells but requires correction of data for cellular multiplicity in cytotoxicity assays. These corrections were performed in the same way as reported.[50,51]

Cytotoxicity assays. HeLa cells from exponentially growing subcultures were plated in triplicate in 25 cm^2 flasks (Becton–Dickinson) at a density of 600–1200 cells per flask, and incubated at 37 °C for 4 h prior to drug

Table 5. Proton NMR data ($CDCl_3$) for selected compounds

Compound	H-8	NH(6)	CH(9)	NH(2)	2×CH_3 isop[b]	Other major signals
5a[a]	8.02 s	—	3.68 s (CH_3)	—	—	5.85 m, 1H (CH=); 5.22–5.14 m, 2H (CH_2=); 4.80 br s, 2H (CH_2); 3.19 br s, 3H (CH_3)
5b	7.67 s	—	4.82 sept (J = 6.79 Hz)	—	1.53 d (J = 6.75)[c]	6.00–5.80 m, 1H (CH=); 5.26–5.17 m, 2H (CH_2=); 4.65 br s (CH_2); 3.37 br s, 3H (CH_3)
5c	7.67 br s	6.30 br t	4.83 sept (J = 6.71 Hz)	—	1.56 d (J = 6.74)	7.40–7.27 m, 5H (Phe)
5d[a]	8.17 s	8.64 br t	4.76 quint (J = 7.32)	—	—	7.33–7.22 m, 5H (Phe); 4.60 br d (**CH_2** Phe); 2.15–1.68 m (4×CH_2)
5e[a]	8.18 s	8.63 br t	4.27 br quint	—	—	7.36–7.21 m, 5H (Phe); 4.60 br d (**CH_2** Phe); 2.05–1.10 m (5×CH_2)
5f	8.18 s	8.71 br t	—	—	—	7.39–7.20 m, 10H (2×Phe); 5.33 s (9-CH_2-Phe); 4.60 br d (NH-**CH_2** Phe), 1H, J = 6.1)
5g[a]	8.17 s	8.64	4.68 m	—-	1.50 d (J = 6.73)	7.76 s (H1′Ph), 1H; 7.58 d 1H, H5′Ph (J = 7.41); 7.37 d 1H H3′Ph (J = 7.72); 7.11 t 1H, H4′Ph (J = 7.80); 4.57 br s, 2H (CH_2)
5h	7.68 s	—	4.80 sept (J = 6.80 Hz)	—	1.54 d (J = 6.74)	5.89–5.75 m 2H (olefin); 4.58 br m (CH_2); 4.36, br m (CH_2); 2.30, br m (CH_2)
6[a]	7.67 s	—	3.55 s (CH_3-N9)	6.16 br t		5.96–5.77 m, 1H (CH=); 5.19–5.00 m, 2H (CH_2=); 4.60, br m, 2H (CH_2); 3.63 m, 2H (**CH_2**OH); 3.39–3.30, m, 5H (NH−**CH_2**, CH_3−N6)
7[a]	7.81 s	—	4.63, m	6.16 br t	1.46 d (J = 6.65)	6.00–5.75 m, 1H (CH=); 5.25–5.75 m 2H (CH_2=); 4.62–4.56 m 2H (CH_2−N6); 3.58–3.51 m, 2H (**CH_2**OH); 3.34 br m, 5H (CH_3-N6, NH-**CH_2**)
8	7.55 s	—	4.63, m	5.20 br t	1.53 d (J = 6.73)	5.92–5.65 m, 2H (CH=CH); 5.15 br m, 1H (OH); 4.57 m, 2H (N−**CH_2**−CH=); 4.34 br t, 2H (N−**CH_2**−CH_2); 3.82 br m, 2H (NH−**CH_2**); 3.57 br m, 2H (**CH_2**OH); 2.28 br m, 2H (N−CH_2−**CH_2**)
9[a]	7.79 s	7.91 br t	4.55 m	—	1.47 d (J = 6.6)	7.40–7.17 m, 5H (Phe); 4.90 br t, 1H (OH); 4.90 br s, 2H (**CH_2**−Ph); 4.05 br m, 1H (**CH**−CH_2OH); 3.65 m, 1H (CH−N); 3.50–3.40 m, 3H (CH−N, **CH_2**OH); 1.96–1.77 m, 4H (CH_2CH_2)
10	7.46 s	6.95 br t	4.59 quint (J = 6.72)	—	1.54 d (J = 6.76)	7.41–7.25 m, 5H (Phe); 6.11 br t, 1H (OH); 4.79 br d, 2H (**CH_2**−Ph); 4.31 m,1H (**CH**−CH_2OH); 3.90–3.55 m, 4H (CH_2−N, **CH_2**OH); 2.21–1.58 m, 4H (**CH_2CH_2**)
11	7.55 s	6.17 br t	4.60 m	—	1.52 d (J = 6.21)	7.37–7.12 m, 10H (2 Phe); 4.91 s, 2H (**CH_2**−Ph); 4.69 br d, 2H (NH−**CH_2**/Ph); 3.80 m, 4H (CH_2CH_2OH); 1.80 s, 1H (OH)
12	7.54 s	6.20 br t	4.60 m	5.55 br t	1.56 d (J = 6.61)	7.51–7.10 m, 10H (2 Phe); 4.98 d, 2H (J = 6.98); 4.81 br m, 1H (**CH**−Ph); 3.85–3.54 m, 2H (NH−**CH_2**−CH); 1.9 br d 1H (OH)
13	7.50 s	6.13 br t	4.60 m	5.20 br d	1.52 d (J = 6.7)	7.45–7.18 m, 5H (Phe); 4.08–3.88 m 1H (**CH**−NH); 3.85–3.51 m (**CH_2**−OH); 1.50–1.15 m, 6H (3 CH_2); 0.96–0.78 br t, 3H (CH_3)
14	7.51 s	6.15 br t	4.60 quint (J = 6.28)	5.77 br d	1.51 d (J = 6.5)	7.45–7.08 m, 10H (2 Phe); 5.19–4.98 m, 1H (**CH**−CH_2OH); 4.90–4.65 br d, 2H (**CH_2** Ph); 4.05–3.81 m, 2H (**CH_2**OH); 2.30 br, 1H (OH)
15	Identical to **14**					
16	7.48 s	6.12 br t	4.60 quint (J = 6.74)	5.19 br d	1.53 d (J = 6.7)	7.40–7.05 m, 10H (2 Phe); 4.76 br d, 2H (**CH_2**−Ph); 3.92–3.52 m, 2H (Ph−**CH_2**−CH); 2.90 dd, 2H (**CH_2**OH) ^2J = 5.06; ^3J = 2.18
17	Identical to **16**					
18	7.46 s	6.11 br t	4.57 quint (J = 6.78)	5.08 br d	1.50 d (J = 6.7)	7.42–7.20 m, 5H (Phe); 4.76 br d, 2H (**CH_2**−Ph); 3.97 br m, 1H (**CH**−NH); 3.86–3.5 m, 3H (**CH_2**OH); 1.65–1.30 m, 4H (CH−**CH_2**−**CH_2**); 1.05–0.81 m, 3H (CH_3)
19	7.48 s	6.15 br t	4.60 quint (J = 6.78)	5.08 br d	1.53 d (J = 6.7)	7.44–7.20 m, 5H (Phe); 4.78 br d, 2H (**CH_2**−Ph); 4.25–4.02 m, 1H (**CH**−CH_2OH); 3.80–3.5 m, 2H (**CH_2**OH); 1.23 d, 3H (CH_3)
20	Identical to **19**					
21[a]	7.79 s	7.96 br t	4.54 br quint	4.90 br d	1.47 d (J = 6.68)	7.79 s, 1H (H1'Ph); 7.55 d, 1H, J = 7.9 (H3′ or H5′Ph); 7.40 d, 1H, J = 7.53 (H5′ or H3′Ph); 7.09 t, 1H, J = 7.67 (H4′Ph); 4.54 br d, 2H (**CH_2**−Ph); 4.05 br m, 1H (**CH**−CH_2OH); 3.75–3.25 m, 4H (CH_2−N, **CH_2**OH); 2.05–1.72 m, 4H (2 CH_2)
22	7.47 s	6.09 br t	4.65 quint (J = 6.71)	—	1.53 d (J = 6.78)	7.45–7.15 m, 5H (Phe); 4.79 br d, 2H (**CH_2**−Ph); 4.54 m, 1H (**CH**OH); 3.53–3.85 m, 4H (CH_2−N−CH_2); 2.40–1.90 m, 3H (OH, CH_2)
23[a]	7.76 s	7.90 br t	4.12 br quint	—	—	7.43–7.13 m, 5H (Phe); 4.94 br t, 1H (OH); 4.60 br d, 2H (**CH_2**−Ph); 4.24–3.97 m, 3H (CH−N, CH_2−N); 3.72–3.58 m, 1H (**CH**−CH_2OH); 3.50–3.37 m, 2H (**CH_2**OH); 2.10–1.60 m, 10H (5 CH_2); 1.55–1.15 m, 4H (2 CH_2)
24[a]	7.74 s	7.87 br t	4.75–4.48 br quint	—	—	7.42–7.10 m, 5H (Phe); 4.88 br t, 1H (OH); 4.75–4.48 m, 2H (**CH_2**−Ph); 4.15–3.97 m, 2H (CH_2−N); 3.72–3.57 m, 1H (**CH**−CH_2OH); 3.53–3.45 m, 2H (**CH_2**OH); 2.18–1.54 m, 12H (6 CH_2)
25[a]	7.83 s	7.94 br t	—	—	—	7.45–7.10 m, 10H (2 Phe); 5.17 s, 2H (**CH_2**−Ph); 4.85 br t, 1H (OH); 4.67 br t, 2H (NH−**CH_2**−Phe); 4.05 br t, 2H (N−CH_2); 3.74–3.6 m, 1H (**CH**−CH_2OH); 3.5–3.35 m, 2H (**CH_2**OH); 2.00–1.72 m, 4H (2 CH_2)

[a] Solvent was DMSO-d_6.
[b] Isopropyl.
[c] Coupling constants J are given in Hertz. s, singlet; d, doublet; t, triplet, q, quartet; quint, quintet; sept, septet; m, multiplet; br, broad.

exposure. Following treatment, the flasks were rinsed and cells returned to normal growth medium for 10–12 days. Colonies were fixed with methanol, stained and scored visually.

All drugs were prepared as 50 mM sterile solutions in pure DMSO and stored at −20 °C for no more than 4 days. Drugs were adjusted extemporarily at the appropriate concentrations by successive dilutions in pure DMSO and in growth medium through vortexing. The final concentration of DMSO was low enough (≤0.5%) as not to alter either cell growth, or drug or radiation response. Exposure to drugs was carried out in dim light to prevent photodegradation of the compounds. Drug was carefully removed through two washes with Hank's balanced saline (37 °C), after which cells were returned to normal growth medium.

γ-Ray irradiation of cells with or without concomitant drug exposure was performed at room temperature (21–24 °C) in medium saturated with 93% air–7% CO_2, using an IBL-637 (^{137}Cs) irradiator (CIS-BioInternational). The dose rate was 0.9 Gy/min.

Thymidine uptake. For studies of altered thymidine intake by drugs, 6×10^5 HeLa cells were seeded in 10 cm^2 Petri dishes and returned to the incubator for 16 h. All dishes at that time contained $1.00\pm0.05\times10^6$ cells. Dishes were washed with warm Hank's buffer, then fed with exactly 2 mL of complete medium and incubated for 90 min with various amounts of the drugs of interest. 2 μCi of [methyl-^{3}H]thymidine were then introduced and dishes allowed to stand in the incubator for a further 30 min. Dishes were then carefully washed twice with Hank's buffer, and cells were lysed with 1 mL lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 0.5% lauryl sulfate, pH 7.8). The lysates were recovered, transferred to scintillation vials and the radioactivity counted by scintillation.

Cytofluorimetric analysis. Cell cycle progression in subcultures and in treated cells was monitored by dual parameter flow cytometry using a FACStarPLUS cytofluorometer (Becton–Dickinson). Cells were incubated with BrdUrd (10 μM, 15 min) for pulse-labeling of S-phase cells, then harvested by trypsinization, pelleted, washed once with cold phosphate-buffered saline and fixed in 70% ice-cold EtOH. Treatment of fixed cells for cytofluorimetric analysis and bivariate data acquisition and processing were done as described.[52]

Data handling. Drug survival fitted a single-exponential model,

$$\frac{S}{S_0} = \exp^{-a[Drug]} \quad (1)$$

where s/s_0 is the surviving fraction relative to the plating efficiency. On the other hand, radiation survival is classically described using a linear-quadratic equation,[53]

$$\ln\frac{S}{S_0} = -\alpha D - \beta D^2 \quad (2)$$

where D is the radiation dose, and α and β numerical parameters characterizing the radiosensitivity of the cell line under consideration.

Results have been expressed as mean ± standard deviation. Least-squares regression analyses were performed using a self-convergent Gauss–Levenberg–Marquardt algorithm (Kaleidagraph 3.0.8, Abelbeck Software Inc.).

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