Synthesis and Evaluation of Organosilicon Inhibitors of Active Purine Transport in Human Osteoblasts

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In the search for new compounds that might, once incorporated into biomaterials, stimulate the natural processes of bone regeneration, a new series of silicon-containing alkyl nucleobase analogues has been synthesized. An active hypoxanthine transport process in human osteoblasts was demonstrated, with an apparent Michaelis constant of 2.3 μM and a maximum possible rate of 0.47 pmol $s^{-1} \times 10^6$ cell. The synthesized analogues were tested for toxicity in human osteoblasts. Nontoxic analogues were tested in competition transport studies with [^{14}C]hypoxanthine. Two of them were found to inhibit the active transport of hypoxanthine in human osteoblasts, with IC_{50} values of 6.5 and 11.6 μM.

KEYWORDS:

bone regeneration · cell recognition · medicinal chemistry · nucleobases · silicon

Introduction

Bone is a living organ undergoing a continuous remodeling process through the action of two types of cells: osteoclasts and osteoblasts. Osteoclasts degrade bone by a process called resorption, releasing calcium and proteins, while osteoblasts are in charge of bone formation. To maintain adequate bone density it is necessary to maintain a perfect working balance of these two processes.[1] In case of a minor bone fracture, only a few weeks are required for bone to repair itself. However, if a large part of the bone has been destroyed, or has been removed by surgery (as in osteosarcoma), the natural healing processes are not sufficient. Implantation of some kind of filler is commonly performed today, with the main purpose of restoring the mechanical properties of the damaged bone. A number of biocompatible materials, such as hydroxyapatite[2] and glass ceramics,[3] have been developed. Even the most advanced of these materials, however, do not allow regeneration and vascularization of the bone. Therefore, materials that could satisfy the mechanical property requirements of natural bone and also participate in the natural process of bone remodeling would be of great importance.[4]

Our objective is to develop molecules that, once covalently linked to a suitable biomaterial, might behave as osteoblast "concentrators" on biomaterial surfaces through specific interaction with membrane components. Osteoblasts could then synthesize extracellular matrix and, afterwards, colonization could favor bone regeneration and growth.

Membrane proteins represent one of the most attractive molecular targets, as they are cell-specific. Among them, carriers seem the most promising ones as they, unlike receptors, do not induce cellular response. Interactions between carriers and a more or less planar biomaterial would leave enough free carriers on cells to ensure sufficient transport of required metabolites. We considered two metabolites: ascorbic acid[5] and nucleic bases. Ascorbic acid has been shown to be essential for the formation and mineralization of bone[6] and necessary for the in vitro differentiation of osteoblastic cells.[7] Concerning nucleobases, transport through the cell membrane is required for some pathways of nucleotide synthesis in mammalian cells; this obviously occurs during proliferative processes. Studies on nucleobase transport have been restricted to a few cell types: human erythrocytes, lymphoma cells, and human vascular endothelial cells.[8] Transport is independent of extracellular cations, and affinities for pyrimidines and purines are of the same order of magnitude. No carrier in osteoblastic cells had, to our knowledge, previously been described.

As we thought that surface modification of biomaterials with analogues of nucleobases might be a good approach to the defined goal, we first decided to check whether nucleobase transport exists in osteoblasts and, if so, whether synthetic moieties could inhibit this transport and therefore exhibit affinity for the protein involved. We present our results here.

Results and Discussion

Purine active transport

Preliminary studies on nucleobase active transport were undertaken with differentiated human bone marrow stromal cells (HBMSCs). Thus, human osteoprogenitor cells were isolated from

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human bone marrow stromal cells cultured in the presence of dexamethasone (10^{-8} M) for two weeks. Osteoblastic phenotype markers (alkaline phosphatase and osteocalcin) were investigated by cytochemistry for alkaline phosphatase activity and by immunostaining with a monoclonal antibody against osteocalcin. Previous results[9] had demonstrated that these cells are able to calcify the matrix after four weeks culturing, even in the absence of β-glycerophosphate. Moreover, they responded to PTH and 1,25$(OH)_2$D3 (PTH = parathormone, 1,25$(OH)_2$D3 = vitamin D active metabolite).[10, 11] Cells arising from the second subculture were taken to test for the existence of a carrier. Two initial substrates used were radioactively labeled [$^{14}C_8$]adenine and [$^{14}C_8$]hypoxanthine. For adenine, no increase in the intracellular radioactivity was detected. However, HBMSCs showed an accumulation of hypoxanthine inside the cells (Figure 1 a); this

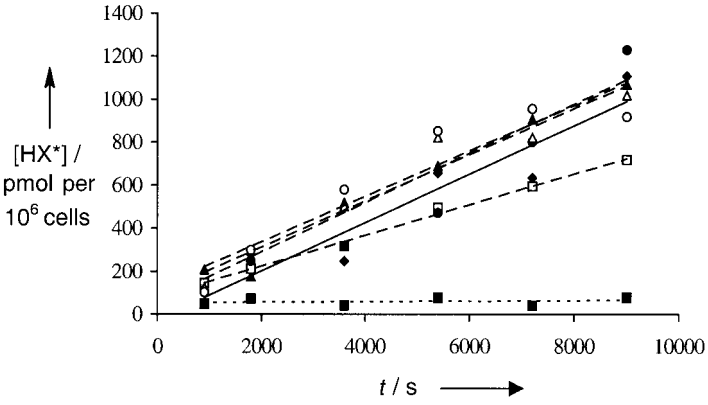


Figure 1. *[^{14}C]Hypoxanthine uptake by human osteoblasts. a) [^{14}C]Hypoxanthine (1 μM, ◆) on its own; b) – f) [^{14}C]hypoxanthine (1 μM) with: b)* ***5 a*** *(100 μM, ▲), c)* ***6*** *(100 μM, □), d)* ***15*** *(100 μM, ●), e)* ***17*** *(100 μM, △), and f)* ***25*** *(100 μM, ○); g) cells incubated with CCCP and NaN_3, and then with [^{14}C]hypoxanthine (1 μM, ■).*

suggests the existence of a transport process. A similar experiment, in which cells had previously been incubated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 200 μM) and NaN_3 (1 mM) to block the energy sources within cells, showed that passive diffusion or facilitated transport were insignificant (Figure 1 g). These results are in clear accordance with the involvement of an active hypoxanthine transport.

To obtain the corresponding transport rates (*k*, neglecting passive diffusion), uptake of [^{14}C]hypoxanthine (HX*) by HBMSCs was measured at different concentrations over a period of time. The hyperbolic plot of [HX*] against *k* shows that the active transport observed reaches a saturation plateau (Figure 2). The mean kinetic hypoxanthine influx parameters in HBMSCs were $K_m = 2.3$ μM and $V_{max} = 0.47$ pmol $s^{-1} \times 10^6$ cells (K_m = the Michaelis constant and V_{max} = the maximum possible rate for the reaction).

Preliminary experiments strongly suggested that this transport was independent of sodium. However, as our main goal was to highlight the presence of a specific carrier in order to focus on this protein as the target of our synthesized derivatives we did not explore these properties any longer.

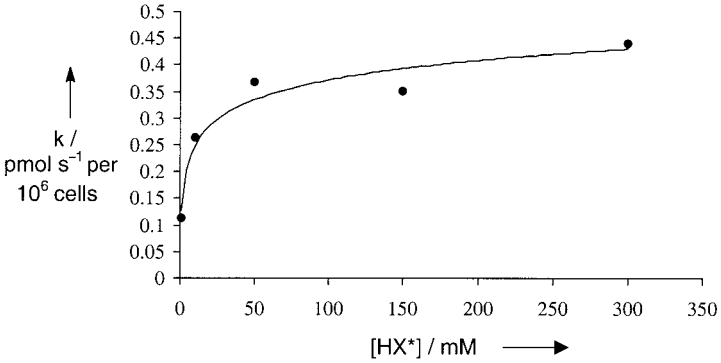
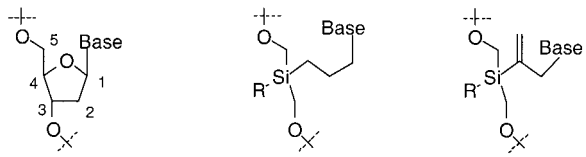


Figure 2. *Concentration dependence of [^{14}C]hypoxanthine (HX*) active transport in human osteoblast cells. The points correspond to the initial rates (k in pmol $s^{-1} \times 10^6$ cells) of hypoxanthine influx into osteoblastic cells measured at extracellular hypoxanthine concentrations ranging from 1 – 300 μM. The kinetic parameters calculated from the plot of 1/k against 1/[HX*] by nonlinear regression are $K_m = 2.3$ μM and $V_{max} = 0.47$ pmol $s^{-1} \times 10^6$ cells.*

Chemistry

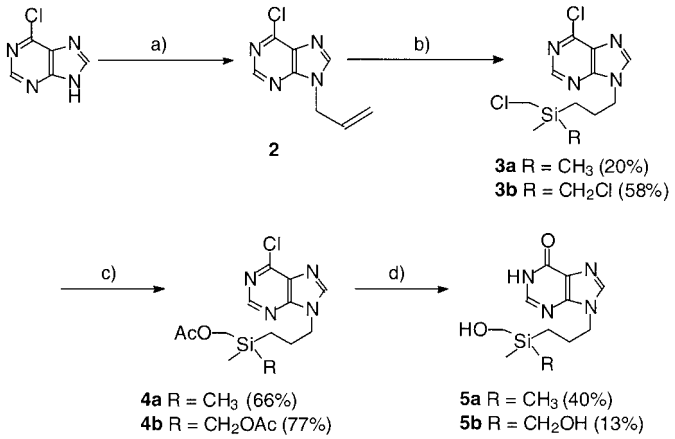
The synthesis substrates chosen were silylalkyled nucleobases, for two reasons. Firstly, acyclo nucleosides have been described in many therapeutic applications (for example, against HIV or cancer) as being recognized by numerous mammalian cell systems and being accessible through easier syntheses. Moreover we have previously reported the synthesis of a series of alkyl- or alkenylsilylated nucleobases as analogues of silylated acyclonucleosides.[12, 13] In these molecules, a silicon atom replaced the carbon-4′ atom of the deoxyribose, while the carbon-2′ and the carbohydrate oxygen atomswere absent (Scheme 1). Among these structures, the presence of a vinylidene moiety appeared to be more favorable and allowed a closer structural resemblance to a natural nucleoside, as evidenced by molecular modeling. Here we report a new and improved synthesis of two unsaturated silyl alkyl nucleobases—namely, 6-chloropurine and hypoxanthine—and their activity towards active purine transport in human osteoblasts.



Scheme 1. *Structural comparison of a natural nucleoside (left) with the proposed acyclic saturated and unsaturated analogues (middle and right, respectively).*

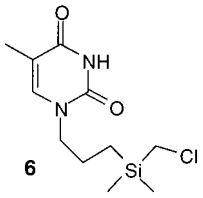
Readily available (chloromethyl)dimethylsilane (**1 a**) and di-(chloromethyl)methylsilane (**1 b**) were prepared by hydride reduction of the corresponding chlorosilanes, and these were used for the key hydrosilylation reaction with the appropriate allylic precursors.[12]

Saturated analogues: Hypoxanthine analogues were synthesized by previously described methods (Scheme 2).[12] Commercially available 6-chloropurine was alkylated with allyl bromide to afford 9-allyl-6-chloropurine (**2**) as the major product in 60%

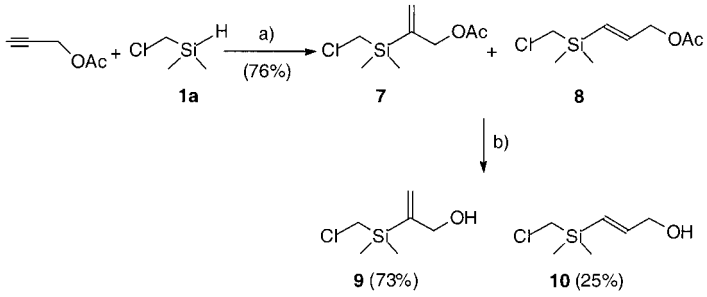


Scheme 2. *Reagents and conditions: a) allyl bromide/NaH (60%)/DMF; b)* ***1a*** *or* ***1b****/$H_2PtCl_6 \cdot H_2O$; c) AcONa/DMF; d) HCl.*

yield. Hydrosilylation with silanes **1a** and **1b** afforded **3a** and **3b**, respectively. Hypoxanthine derivatives **5a** and **5b** were obtained after acetoxylation followed by treatment in refluxing HCl. The thymine analogue 1-{[3-(chloromethyl)dimethylsilyl]-propyl}-5-methyl-*1H*-pyrimidine-2,4-dione (**6**) has been described previously.[12]

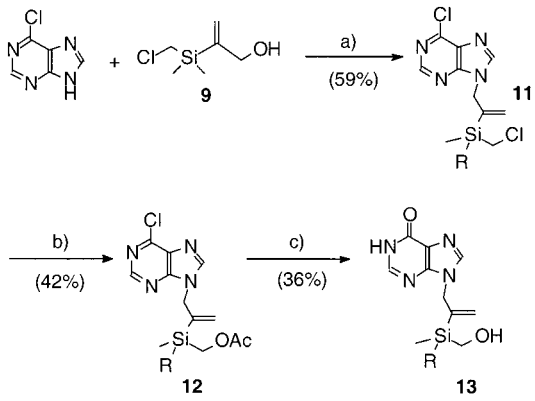


Unsaturated analogues: Hydrosilylation of propargyl acetate with **1a** afforded a mixture of isomers **7** and **8**, which could not be separated. Acid methanolysis of the mixture gave the corresponding alcohols **9** and **10**, which were separated by silica gel chromatography (Scheme 3).



Scheme 3. *Reagents and conditions: a) $H_2PtCl_6 \cdot H_2O$/THF; b)* para*-toluenesulfonic acid/MeOH.*

The desired alcohol was treated with the appropriate heterocycle in the presence of Ph_3P and DEAD (diethylazodicarboxylate) in a modification of the Mitsunobu reaction, to afford the 9-alkylated heterocycle.[14] Scheme 4 shows the general synthetic strategy with 6-chloropurine and vinylidene alcohol to yield **11**, and its acetoxylation followed by hydrolysis to afford compound **13**.



Scheme 4. *Reagents and conditions: a) Ph_3P/DEAD; b) NaOAc/DMF; c) 1 N HCl.*

The adenine analogues **18** and **20** were prepared in a similar manner. In place of unprotected thymine, 3-*N*-benzoylthymine (**21**) was used in the Mitsunobu reaction to yield analogues **23** and **25**. The hypoxanthine derivative **14** was obtained from intermediate compound **11**, by acid hydrolysis with trifluoroacetic acid, and the thio derivative **15** was obtained by treatment of **11** with thiourea. The mercaptopurine derivative **17** was obtained from **12** by treatment with thiourea followed by basic methanolysis (K_2CO_3). The synthesis of these compounds is summarized in Table 1.

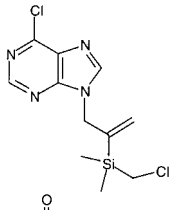
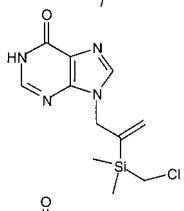
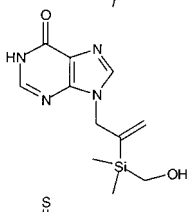
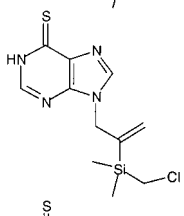
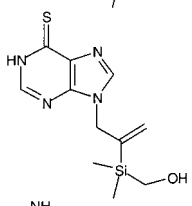
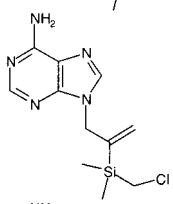
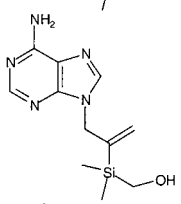
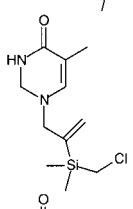
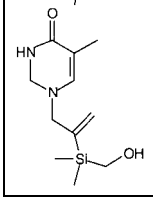
Biological assays

Cytotoxicity: Although the final intended application of the synthesized molecules is incorporation into solid supports (biomaterials), their cytotoxicity towards osteoblasts in solution was tested in vitro by a cell metabolic activity assay (MTT assay).[11] From the results obtained (Figure 3), molecules **3a**, **3b**, **11**, **18**, **20**, and **23** could be considered cytotoxic. Compounds **5a**, **5b**, **6**, **13**, **14**, **15**, **17**, and **25** could be considered nontoxic, since the observed cell viable percentage was over 75% relative to the control.

Active transport inhibition test: All nontoxic compounds were tested in competition transport studies with [^{14}C]hypoxanthine in HBMSCs. A compound was considered to inhibit hypoxanthine transport if it induced a decrease in the intracellular accumulation of [^{14}C]hypoxanthine. Of the molecules tested, compounds **5a**, **6**, **15**, **17**, and **25** were inactive (Figure 1b–f). Compounds **13** and **14**, however, showed good inhibition of the active transport of hypoxanthine in human osteoblasts (Figure 4b, c). For these two analogues, a series of assays were performed that allowed us to determine IC_{50} values of 11.6 ± 1.7 and 6.5 ± 1 μM for **13** and **14**, respectively (IC_{50} = the concentration required for 50% inhibition).

In conclusion, we have observed, to our knowledge, for the first time the uptake of hypoxanthine in human osteoblasts, while no incorporation was observed for adenine. This uptake is energy-dependent, and the amount of passive diffusion is negligible. This strongly suggests that hypoxanthine is involved in an active transport process with kinetics parameters of $K_m = 2.3$ μM and $V_{max} = 0.37$ pmol $s^{-1} \times 10^6$ cells. The study of this

Table 1. *Synthesis of silylalkenyl nucleobases.*

Compound		Starting material	Conditions
	**11**	6-chloro-purine	**9a**, Ph_3P, DEAD
	**13**	**11**	1) NaOAc/DMF; 2) HCl/H_2O
	**14**	**11**	CF_3CO_2H/H_2O
	**15**	**11**	thiourea/propanol
	**17**	**12**	1) thiourea/propanol; 2) K_2CO_3/MeOH
	**18**	adenine	**9a**, Ph_3P, DEAD
	**20**	**18**	1) NaOAc/DMF; 2) K_2CO_3/MeOH
	**23**	**21**	1) **9a**, Ph_3P, DEAD; 2) NH_4OH/MeOH
	**25**	**23**	1) NaOAc/DMF; 2) *para*-toluenesulfonic acid/MeOH

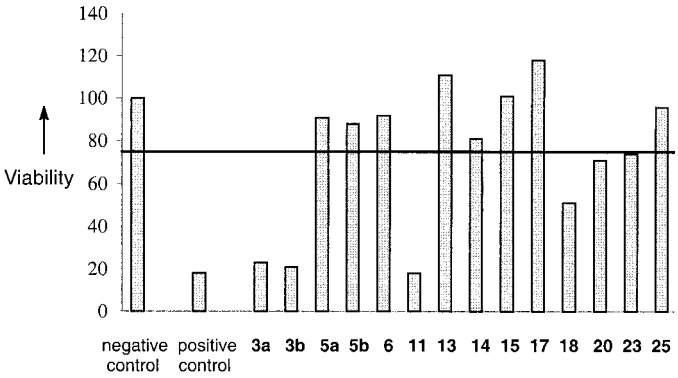


Figure 3. *Histogram representing the results of the MTT cytotoxicity assay for compounds incubated for 24 h at a 1 mM concentration. The negative control is the culture medium (IMDM), and the positive control is the culture medium plus phenol (64 g L^{-1}). Viability is expressed in terms of the percentage of the negative control. Compounds displaying viabilities above 75% (horizontal line) are considered nontoxic.*

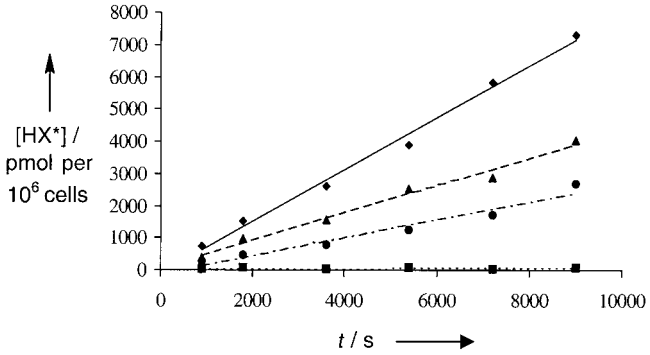


Figure 4. *Effect of derivatives **13** and **14** on the uptake of [^{14}C]hypoxanthine. a) [^{14}C]Hypoxanthine (2 μM, ◆) on its own; b) [^{14}C]hypoxanthine (2 μM) with **13** (150 μM, ▲); c) [^{14}C]hypoxanthine with **14** (150 μM, ●); d) cells incubated with CCCP and NaN_3, and then with [^{14}C]hypoxanthine (1 μM, ■).*

molecular recognition event by a specific carrier is a good target for the design of a biomaterial promoting osteoblast concentration and thus allowing the initial steps of their adhesion. We have synthesized a series of saturated and unsaturated silylalkyl and silylalkenyl nucleobases, and their cytotoxicity towards osteoblasts has been evaluated in vitro. It is interesting to note that toxicity depends very greatly on the nature of the purine ring, and very little on the acyclic moiety, regardless of whether the latter is saturated or unsaturated or possesses halogen or hydroxy substituents. Noncytotoxic compounds **5a**, **6**, **13**, **14**, **15**, **17**, and **25** were tested in competition transport assays with [^{14}C]hypoxanthine. Compounds **13** and **14** were found to inhibit [^{14}C]hypoxanthine uptake in human osteoblasts with IC_{50} values of 11.6 ± 1.7 and 6.5 ± 1 μM, respectively. It is plausible that these molecules inhibit hypoxanthine uptake by specifically binding to the carrier proteins. If that is the case, these molecules, when attached to biomaterials, should provide a point of anchorage to act as concentrators of osteoblasts. This should favor the presence of these cells around the biomaterial, which might undergo the secretion of extracellular matrix and bone neoformation.

Experimental Section

General: NMR spectra were recorded at 200 MHz (^{1}H) or 50 MHz (^{13}C) at 298 K on a Bruker AC 200 spectrometer. Chemical shifts are expressed in parts per million downfield from tetramethylsilane (TMS). Mass spectra were obtained under fast atom bombardment (FAB) conditions (LRMS: Finnigan Mat TSQ70 spectrometer, HRMS: VG Analytical Autospec EQ). Elemental analyses were performed by the "Service Central d'Analyse", CNRS, Vernaison (France). Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Tetrahydrofuran (THF) was distilled over potassium/benzophenone immediately before use; *N*,*N*-dimethylformamide (DMF) was distilled over CaH_2; methanol was distilled over magnesium. Dry sodium acetate was obtained by heating the solid on a hot porcelain plate until it melted and the water had evaporated. The solid was then recovered after cooling and used immediately. Unless otherwise stated, reactions were performed under nitrogen atmosphere and the crude reaction mixtures were treated as follows: the organic solution was washed with water, neutralized if necessary, separated from the aqueous layer, and then dried over magnesium sulfate. The organic solvent was evaporated under vacuum to afford the crude product. Column chromatography was performed on silica gel 60 (70–230 mesh). Flash column chromatography was performed on silica gel 60 (230–400 mesh) at 0.4 bar. Thin layer chromatography (TLC) was performed on aluminium plates precoated with silica gel 60 F_{254} and viewed either under UV light or with the aid of 1% $KMnO_4$ in water.

Tissue culture plates were purchased from Nunc, Cyclopore was from Falcon or Becton-Dickinson. Cell culture medium (IMDM: Iscove's modified Dulbecco's medium) was supplied by GIBCO. Fetal calf serum (FCS) and trypsin were purchased from Boehringer Mannheim.

Compounds **1a**, **1b**, **2**, **3a**, **3b**, **4b**, and **6** were synthesized according to previously published procedures.[12, 13]

Chemical synthesis:

(Acetoxymethyl)dimethyl-3-[*N*-9-(6-chloropurinyl)]propylsilane (4a): A mixture of **3a** (0.64 g, 2.1 mmol) and dry sodium acetate (0.52 g, 6.3 mmol) in dry DMF was stirred at 120 °C overnight. The solvent was removed under vacuum, and the residue was applied to a silica gel column and eluted with toluene/ethyl acetate (10:90) to afford **4a** (0.46g, 66%). ^{1}H NMR ($CDCl_3$): $\delta = 0.01$ (s, 6H; $Si(CH_3)_2$), 0.52 (m, 2H; $SiCH_2CH_2$), 1.91 (m, 2H; $SiCH_2CH_2$), 1.93 (s, 3H; $OCOCH_3$), 3.68 (s, 2H; $SiCH_2O$), 4.22 (t, $J = 7$ Hz, 2H; $SiCH_2CH_2CH_2N$), 8.08 (s, 1H; H-8), 8.66 (s, 1H; H-2); ^{13}C NMR ($CDCl_3$): $\delta = -5.19$, 10.65, 20.41, 24.02, 47.02, 55.98, 131.24, 145.13, 150.49, 151.49, 151.74, 171.30.

(Hydroxymethyl)dimethyl-3-[*N*-9-(6-oxopurinyl)]propylsilane (5a): A mixture of **4a** (0.43 g, 1.3 mmol) in 1 N aqueous HCl (5 mL) was heated under reflux for 5 h. The cooled solution was neutralized by addition of 1 N aqueous NaOH and evaporated to dryness. The crude product was taken up in isopropyl alcohol and filtered, and the filtrate was concentrated to give **5a** as a white solid (216 mg, 61%). ^{1}H NMR (CD_3OD): $\delta = -0.04$ (s, 6H; $Si(CH_3)_2$), 0.47 (m, 2H; $SiCH_2CH_2$), 1.84 (m, 2H; $SiCH_2CH_2$), 4.15 (t, $J = 7$ Hz, 2H; $SiCH_2CH_2CH_2N$), 4.79 (s, 2H; $SiCH_2O$), 7.97 (s, 1H; H-8), 7.99 (s, 1H; H-2); ^{13}C NMR (CD_3OD): $\delta = -5.15$, 11.31, 25.81, 47.73, 54.58, 142.20, 146.73, 150.26, 159.42, 180.34; HRMS: calcd for $C_{11}H_{18}N_4O_2Si$: 267.1277; found: 267.1276.

Bis(hydroxymethyl)methyl-3-[*N*-9-(6-oxopurinyl)]propylsilane (5b): By the above procedure, compound **4b** (0.21 g, 0.5 mmol) was refluxed with 1 N aqueous HCl for 5 h to give **5b** as a white solid (20 mg, 13%). ^{1}H NMR (D_2O): $\delta = 0.10$ (s, 3H; $SiCH_3$), 0.67 (m, 2H; $SiCH_2CH_2$), 1.95 (m, 2H; $SiCH_2CH_2$), 4.25 (t, $J = 7$ Hz, 2H; $SiCH_2CH_2CH_2N$), 4.75 (s, 4H; $Si(CH_2)_2O$), 8.12 (s, 1H; H-8), 8.2 (s, 1H; H-2); ^{13}C NMR (D_2O) $\delta = -9.40$, 6.78, 23.71, 47.12, 51.26, 142.41, 145.50, 148.34, 160.01, 179.74; HRMS: calcd: 283.1226; found: 283.1231.

2-[(Chloromethyl)dimethylsilyl]-2-propenyl acetate (7) and (*E*)-3-[(chloromethyl)dimethylsilyl]-2-propenyl acetate (8): A solution of hexachloroplatinic acid in 2-propanol (0.1 M, 0.1 mL) was added to a stirred solution of propargyl acetate (2.94 g, 30 mmol) and **1a** (3.58 g, 33 mmol) in dry THF (15 mL). After this had been heated for 12 h at 50 °C, the solvent was removed and the residue was distilled off to afford a mixture of **7** and **8** as a colorless liquid (4.75 g, 76%). bp 53 °C/0.5 mmHg; ^{1}H NMR ($CDCl_3$): $\delta = 0.20$ (s, 6H; $Si(CH_3)_2$), 0.25 (s, 6H; $Si(CH_3)_2$), 2.07 (s, 3H; $OCOCH_3$), 2.08 (s, 3H; $OCOCH_3$), 2.79 (s, 2H; CH_2Cl), 2.85 (s, 2H; CH_2Cl), 4.60 (d, $J = 4$ Hz, 2H; CH_2O in **8**), 4.70 (s, 2H; CH_2O in **7**), 5.52 (m, 1H; C=CH_2 in **7**), 5.89 (m, 1H; C=CH_2 in **7**), 5.95 (d, $J = 19$ Hz, 1H; SiC*H*=CH in **8**), 6.17 (td, $J = 19$, 4 Hz, 1H; SiCH=C*H* in **8**).

2-[(Chloromethyl)dimethylsilyl]-2-propenol (9) and (*E*)-3-[(chloromethyl)dimethylsilyl]-2-propenol (10): *p*-Toluenesulfonic acid (0.46 g, 27 mmol) was added to a solution of **7** and **8** (4.75 g, 23 mmol) in methanol (120 mL). After this had been stirred at reflux for 12 h, the solvent was removed and the residue was applied to a silica gel column and eluted with hexane/ethyl acetate (9:1) to give the desired compounds as colorless liquids. The vinylidene isomer **9** was eluted first, closely followed by **10**. The appropriate fractions were combined and evaporated to give **9** (2.76 g, 72%) and **10** (0.96 g, 25%). Compound **9**: ^{1}H NMR ($CDCl_3$): $\delta = 0.22$ (s, 6H; $Si(CH_3)_2$), 1.83 (s, 1H; O*H*), 2.85 (s, 2H; $SiCH_2Cl$), 4.25 (d, $J = 4$ Hz, 2H; CH_2OH), 5.46 (m, 1H; C=CH_2), 5.85 (m, 1H; C=CH_2); ^{13}C NMR ($CDCl_3$): $\delta = -4.92$, 29.54, 66.30, 125.53, 148.09. Compound **10**: ^{1}H NMR ($CDCl_3$): $\delta = 0.20$ (s, 6H; $Si(CH_3)_2$), 1.70 (s, 1H; O*H*), 2.80 (s, 2H; $SiCH_2Cl$), 4.20 (dd, $J = 4$, 2 Hz, 2H; CH_2O), 5.90 (td, $J = 19$, 2 Hz, 1H; SiC*H*=CH), 6.28 (td, $J = 19$, 4 Hz, 1H; SiCH=C*H*); ^{13}C NMR ($CDCl_3$): $\delta = -4.60$, 30.26, 64.94, 124.46, 147.66.

6-Chloro-9-*N*-{2-[(chloromethyl)dimethylsilyl]-2-propenyl}purine (11): DEAD (0.557 g, 3.2 mmol) was added dropwise at −20 °C over a period of 4 h to a solution of 6-chloropurine (0.5 g, 3.2 mmol), triphenylphosphine (0.85 g, 3.2 mmol), and **9** (0.44 g, 2.7 mmol) in dry THF (15 mL). The mixture was stirred at −20 °C for 72 h. After concentration, the residue was purified on a silica gel column eluted with hexane/ethyl acetate (6:4) to give **11** as a yellow oil (0.43 g, 59%). ^{1}H NMR ($CDCl_3$): $\delta = 0.23$ (s, 6H; $Si(CH_3)_2$), 2.76 (s, 2H; $SiCH_2Cl$), 5.02 (s, 2H; CH_2N), 5.50 (m, 1H; C=CH_2), 5.64 (m, 1H; C=CH_2), 8.07 (s, 1H; H-8), 8.73 (s, 1H; H-2); ^{13}C NMR ($CDCl_3$): $\delta = -4.97$, 29.07, 48.18, 129.68, 131.13, 142.47, 145.42, 150.86, 151.60, 151.95; HRMS: calcd: 301.0443; found: 301.0440.

6-Chloro-9-*N*-{2-[(acetoxymethyl)dimethylsilyl]-2-propenyl}purine (12): A mixture of **11** (0.77 g, 2.5 mmol) and dry sodium acetate (0.63 g, 7.6 mmol) in dry DMF (15 mL) was stirred at 120 °C overnight. The solvent was evaporated under vacuum, and the residue was applied to a silica gel column and eluted with toluene/ethyl acetate (1:9) to give **12** as a yellow oil (0.35 g, 42%). ^{1}H NMR ($CDCl_3$): $\delta = 0.15$ (s, 6H; $Si(CH_3)_2$), 2.01 (s, 3H; $OCOCH_3$), 3.82 (s, 2H; $SiCH_2O$), 5.02 (s, 2H; CH_2N), 5.40 (m, 1H; C=CH_2), 5.58 (m, 1H; C=CH_2), 8.10 (s, 1H; H-8), 8.73 (s, 1H; H-2); ^{13}C NMR ($CDCl_3$): $\delta = -5.28$, 20.46, 47.92, 56.67, 128.65, 130.96, 142.78, 145.65, 150.49, 151.57, 151.71, 171.16.

6-Oxo-9-*N*-{2-[(hydroxymethyl)dimethylsilyl]-2-propenyl}purine (13): A solution of **12** (0.21 g, 0.6 mmol) in 1 N aqueous HCl (6 mL) was heated under reflux for 5 h. The cooled solution was neutralized by addition of 1 N aqueous NaOH and evaporated to dryness. The crude product was taken up in isopropyl alcohol and filtered, and the filtrate was evaporated to give a solid, which was crystallized from water to afford **13** as a white solid (61.2 mg, 36%). ^{1}H NMR (CD_3OD): $\delta = 0.23$ (s, 6H; $Si(CH_3)_2$), 3.45 (s, 2H; $SiCH_2O$), 5.10 (s, 2H; CH_2N), 5.48

(m, 1H; C=CH_2), 5.69 (m, 1H; C=CH_2), 8.10 (s, 1H; H-8), 8.13 (s, 1H; H-2); ^{13}C NMR (CD_3OD): $\delta = -3.67$, 49.33, 55.88, 126.52, 129.72, 144.10, 147.82, 148.27, 151.80, 160.58, 180.26; HRMS: calcd for $C_{11}H_{16}N_4O_2Si$: 265.1121; found: 265.1126.

6-Oxo-9-*N*-{2-([chloromethyl)dimethylsilyl]-2-propenyl}purine (14): A solution of **11** (0.25 g, 0.8 mmol) in 8 mL of CF_3CO_2H/H_2O (3:1) was stirred at room temperature for two d. The solvent was evaporated, and the residue was taken up in MeOH/NH_4OH (3:2) and adjusted to pH 7. After evaporation, the remaining solid was purified on a silica gel column eluted with CH_2Cl_2/MeOH (9:1) to give **14** as a white solid (0.12 g, 56%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.13$ (s, 6H; Si(CH_3)$_2$), 2.96 (s, 2H; SiCH_2Cl), 4.89 (s, 2H; CH_2N), 5.34 (m, 1H; C=CH_2), 5.53 (m, 1H; C=CH_2), 8.00 (s, 1H; H-8), 8.03 (s, 1H; H-2), NH not seen; ^{13}C NMR ([D$_6$]DMSO): $\delta = -5.10$, 29.36, 47.35, 123.80, 128.08, 143.64, 145.71, 148.39, 156.69, 178.83; elemental analysis: calcd (%) for $C_{11}H_{15}ClN_4OSi$ (282.9): C 46.12, H 5.35, N 19.81; found: C 46.97, H 5.24, N 19.47.

6-Thio-9-*N*-{2-[(chloromethyl)dimethylsilyl]-2-propenyl}purine (15): A mixture of **11** (0.20 g, 0.7 mmol) and thiourea (0.06 g, 0.8 mmol) in 2-propanol (20 mL) was heated under reflux for 3 h. After cooling, the solid formed during heating was filtered off and dried under vacuum to afford **15** as a white solid (0.11 g, 58%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.16$ (s, 6H; Si(CH_3)$_2$), 2.99 (s, 2H; SiCH_2Cl), 4.93 (s, 2H; CH_2N), 5.32 (m, 1H; C=CH_2), 5.56 (m, 1H; C=CH_2), 8.21 (s, 1H; H-8), 8.22 (s, 1H; H-2), NH not seen; ^{13}C NMR ([D$_6$]DMSO): $\delta = -5.04$, 26.38, 47.38, 128.06, 134.82, 143.33, 144.13, 145.10, 145.15, 175.91; LRMS: 299 $[M]^+$; elemental analysis: calcd (%) for $C_{11}H_{15}ClN_4SSi$ (298): C 44.29, H 5.07, N 18.79; found: C 43.18, H 4.99, N 18.79.

6-Thio-9-*N*-{2-[(acetoxymethyl)dimethylsilyl]-2-propenyl}purine (16): A solution of **12** (0.55 g, 1.7 mmol) and thiourea (0.15 g, 2 mmol) in 2-propanol (20 mL) was heated under reflux for 2 h and then treated as above to afford **16** as a white solid (0.38 g, 70%). ^{1}H NMR (CD_3OD): $\delta = 0.26$ (s, 6H; Si(CH_3)$_2$), 2.07 (s, 3H; OCOCH_3), 3.90 (s, 2H; SiCH_2O), 5.09 (s, 2H; CH_2N), 5.56 (m, 1H; C=CH_2), 5.71 (m, 1H; C=CH_2), 8.20 (s, 1H; H-8), 8.22 (s, 1H; H-2); ^{13}C NMR (CD_3OD): $\delta = -5.06$, 20.51, 47.86, 55.41, 127.63, 134.90, 143.28, 143.73, 144.12, 145.09, 170.93, 176.24; LRMS: 323 $[M]^+$.

6-Thio-9-*N*-{2-[(hydroxymethyl)dimethylsilyl]-2-propenyl}purine (17): A solution of **16** (0.2 g, 0.6 mmol) and potassium carbonate (0.13 g, 0.9 mmol) in dry methanol (15 mL) was stirred at 40 °C overnight. After concentration, the oily residue was taken up in hot water to give **17** as a white solid (0.077 g, 45%). ^{1}H NMR (CD_3OD): $\delta = 0.05$ (s, 6H; Si(CH_3)$_2$), 3.19 (s, 2H; SiCH_2OH), 4.91 (s, 2H; CH_2N), 5.19 (m, 1H; C=CH_2), 5.46 (m, 1H; C=CH_2), 8.17 (s, 1H; H-8), 8.19 (s, 1H; H-2); ^{13}C NMR (CD_3OD): $\delta = -5.17$, 47.72, 51.92, 126.16, 134.81, 143.36, 144.17, 145.10, 145.16, 175.89; elemental analysis: calcd (%) for $C_{11}H_{16}N_4OSSi$ (280): C 47.13, H 5.76, N 19.0; found: C 46.21, H 5.61, N 20.58.

9-*N*-{2-[(Chloromethyl)dimethylsilyl]-2-propenyl}adenine (18): A solution of **9** (500 mg, 3.0 mmol) and DEAD (557 mg, 3.2 mmol) in THF (10 mL) was added dropwise over 30 min to a suspension of adenine (474 mg, 3.1 mmol) and triphenylphosphine (839 mg, 3.2 mmol) in THF (15 mL). The mixture was stirred for 12 h at room temperature and evaporated. The residue was purified on a silica gel column eluted with CH_2Cl_2/MeOH (98:2) to give **18** as a white solid (241 mg, 28%) after washing with cold carbon tetrachloride. ^{1}H NMR ($CDCl_3$): $\delta = 0.18$ (s, 6H; Si(CH_3)$_2$), 2.76 (s, 2H; SiCH_2Cl), 4.92 (s, 2H; CH_2N), 5.57 – 5.62 (2 × m, 2H; C=CH_2), 5.66 (br s, 2H; NH_2), 7.75 (s, 1H; H-8), 8.36 (s, 1H; H-2); ^{13}C NMR ($CDCl_3$): $\delta = -5.11$, 20.67, 47.66, 119.34, 128.59, 140.72, 143.51, 150.09, 153.14, 155.49, LRMS: 281.3 $[M]^+$.

9-*N*-{2-[(Acetoxymethyl)dimethylsilyl]-2-propenyl}adenine (19): A mixture of **18** (195 mg, 0.69 mmol), freshly dried sodium acetate (120 mg, 1.46 mmol), and dry DMF (8 mL) was stirred at 120 °C for 8 h. The solvent was coevaporated with added toluene and the residue applied to a silica gel column and eluted with CH_2Cl_2/MeOH (95:5) to give **19** as a light yellow solid (150 mg, 71%). ^{1}H NMR ($CDCl_3$): $\delta = 0.13$ (s, 6H; Si(CH_3)$_2$), 2.00 (s, 3H; OCOCH_3), 3.80 (s, 2H; SiCH_2O), 4.91 (s, 2H; CH_2N), 5.45 and 5.55 (2 × m, 2H; C=CH_2), 5.87 (br s, 2H; NH_2), 7.76 (s, 1H; H-8), 8.34 (s, 1H; H-2); ^{13}C NMR ($CDCl_3$): $\delta = -5.04$, 20.72, 47.71, 56.01, 119.38, 128.63, 140.76, 143.55, 150.13, 153.19, 155.52, 171.53.

9-*N*-{2-[(Hydroxymethyl)dimethylsilyl]-2-propenyl}adenine (20): Potassium carbonate (70 mg, 0.50 mmol) was added to a solution of **19** (150 mg, 0.49 mmol) in methanol (15 mL). The reaction mixture was stirred at room temperature for 12 h, and 1 N aqueous HCl (1 mL) was then added. The resulting solution was concentrated to dryness and the remaining solid was purified on a silica gel column eluted with CH_2Cl_2/MeOH (9:1) to give **21** as a white, amorphous solid (101 mg, 78%). ^{1}H NMR (CD_3OD): $\delta = 0.03$ (s, 6H; Si(CH_3)$_2$), 3.29 (s, 2H; SiCH_2OH), 4.82 (s, 2H; CH_2N), 5.18 and 5.44 (2 × m, 2H; C=CH_2), 7.76 (s, 1H; H-8), 8.13 (s, 1H; H-2); ^{13}C NMR (CD_3OD): $\delta = -5.83$, 47.85, 53.61, 118.50, 127.23, 141.21, 143.97, 149.02, 151.86, 155.50; LRMS 263.3 $[M]^+$.

1-*N*-{2-[(Chloromethyl)dimethylsilyl]-2-propenyl}-3-*N*-benzoylthymine (22): A solution of **9** (500 mg, 3 mmol) and DEAD (1.30 g, 7.5 mmol) in THF (12 mL) was added dropwise over 1 h to a cold (0 °C) suspension of **21** (1.15 g, 5 mmol) and triphenylphosphine (1.97 g, 7.5 mmol) in THF (8 mL). The mixture was stirred for 20 h at 0 °C and evaporated. The residue was purified on a silica gel column eluted with CH_2Cl_2/MeOH (100:0, then 99:1) to give **22** as a colorless oil that slowly crystallized upon standing for a few hours (700 mg, 62%). mp 107 °C; ^{1}H NMR ($CDCl_3$): $\delta = 0.25$ (s, 6H; Si(CH_3)$_2$), 1.95 (d, $^4J = 1$ Hz, 3H; thymine CH_3), 2.83 (s, 2H; SiCH_2Cl), 4.47 (s, 2H; CH_2N), 5.67 and 5.77 (2 × m, 2H; C=CH_2), 7.00 (d, $J = 1$ Hz, 1H; H-6), 7.50 – 7.93 (m, 5H; benzoyl arom-H); ^{13}C NMR ($CDCl_3$): $\delta = -4.81$, 12.38, 29.30, 51.92, 111.02, 129.12, 129.57, 130.39, 131.61, 134.94, 139.58, 142.70, 149.74, 163.05, 168.84.

1-*N*-{2-[(Chloromethyl)dimethylsilyl]-2-propenyl}thymine (23): A solution of **22** (700 mg, 1.85 mmol) in methanol (60 mL) and 5% aqueous ammonia solution (8 mL) was stirred at room temperature until disappearance of the starting material as monitored by TLC (CH_2Cl_2/MeOH 98:2). After concentration, the residue was purified on a silica gel column eluted with CH_2Cl_2/MeOH (95:5) to give **23** as a white solid (420 mg, 83%). mp 144 °C; ^{1}H NMR ($CDCl_3$): $\delta = 0.22$ (s, 6H; Si(CH_3)$_2$), 1.88 (d, $J = 1.2$ Hz, 3H; thymine CH_3), 2.82 (s, 2H; SiCH_2Cl), 4.43 (s, 2H; CH_2N), 5.58 and 5.67 (2 × m, 2H; C=CH_2), 6.88 (d, $J = 1.2$ Hz, 1H; H-6), 9.70 (s, 1H; NH); ^{13}C NMR ($CDCl_3$): $\delta = -4.86$, 12.29, 29.37, 51.56, 110.96, 129.21, 139.85, 142.92, 150.98, 164.45; LRMS: 272.3 $[M]^+$.

1-*N*-{2-[(Acetoxymethyl)dimethylsilyl]-2-propenyl}thymine (24): A mixture of **23** (390 mg, 1.4 mmol), dry sodium acetate (260 mg, 3.1 mmol), and DMF (10 mL) was stirred at 120 °C for 8 h. The solvent was coevaporated with added toluene, and the residue was purified on a silica gel column eluted with hexane/ethyl acetate (3:7) to give **24** as a colorless oil (300 mg, 70%). ^{1}H NMR ($CDCl_3$): $\delta = 0.19$ (s, 6H; Si(CH_3)$_2$), 1.90 (d, $J = 1$ Hz, 3H; thymine CH_3), 2.02 (s, 3H; OCOCH_3), 3.85 (s, 2H; SiCH_2O), 4.44 (s, 2H; CH_2N), 5.50 and 5.60 (2 × m, 2H; C=CH_2), 6.90 (d, $J = 1$ Hz, 1H; H-6), 8.51 (br s, 1H; NH); ^{13}C NMR ($CDCl_3$): $\delta = -5.19$, 12.13, 20.56, 51.22, 55.93, 110.62, 127.83, 139.94, 143.07, 150.95, 164.51, 171.41.

1-*N*-{2-[(Hydroxymethyl)dimethylsilyl]-2-propenyl}thymine (25): A solution of **24** (300 mg, 1 mmol) and potassium carbonate

(140 mg, 1 mmol) in methanol (15 mL) was stirred at room temperature until completion of the reaction (approximately 24 h) as monitored by TLC (CH_2Cl_2/MeOH 96:4). The mixture was acidified with 1 N HCl (1 mL), and the resulting solution was concentrated. The residue was purified on a silica gel column eluted with CH_2Cl_2/MeOH (90:10) to give **25** as a colorless oil (240 mg, 94%). ^{1}H NMR (CD_3OD): $\delta = 0.16$ (s, 6H; Si(CH_3)$_2$), 1.86 (d, $J = 1.3$ Hz, 3H; thymine CH_3), 3.39 (s, 2H; SiCH_2OH), 4.83 (s, 2H; CH_2N), 5.50 and 5.60 (2 × m, 2H; C=CH_2), 7.28 (d, $J = 1.3$ Hz, 1H; H-6); ^{13}C NMR (CD_3OD): $\delta = -3.57$, 13.80, 54.40, 56.00, 112.89, 129.08, 144.58, 147.57, 154.44, 168.43; HRMS: calcd: 255.1164, found: 255.1162.

Biological evaluation:

Human bone marrow stromal cells culture: Human bone marrow was obtained by aspiration from the femoral diaphysis or iliac from patients (aged 20 – 70 years) undergoing hip prosthesis surgery after trauma. Cell culture was initiated in the presence of Dexamethasone at 10^{-8} M and was performed as described previously,[11] with some modifications. HBMSCs were used from the second to the sixth passages.

Alkaline phosphatase activity measurement: Intracellular alkaline phosphatase activity (Al-P) was determined at confluency, as described by Majeska and Rodan,[15] arising from the different culture conditions. The medium was removed and the cells were washed twice with 0.1 M phosphate buffered sline (PBS; pH 7.4), and then scraped and sonicated for 30 seconds at 20 kHz and 8% amplitude (Vibra-Cell, Bioblock-Scientific) in 0.1 M PBS (200 µL; pH 7.4). The release of *p*-nitrophenol from 10 mM *p*-nitrophenyl phosphate in a buffer containing 1 mM $MgCl_2 \cdot 6H_2O$ in 2-amino-methyl-1-propanol (pH 10.3) was measured at 37 °C. The reaction was stopped with 0.1 N NaOH after 30 min, and the absorbance was determined at 405 nm by a spectrophotometer (Spectronic Genesis 5 S, Bioblock Scientific, Spectronic Instruments (Rochester, NY)). Data are expressed as a ratio of nmol of inorganic phosphate (Pi) cleaved by the enzyme in 30 min per µg protein. The quantitative measurements of cellular proteins were performed by Lowry's method as previously described.[10]

Osteocalcin immunostaining: Cells arising from the second subculture were plated at 1×10^4 cells cm^{-2} in Lab-tek chamber slides in IMDM supplemented with 10% FCS. Three days later, the cell layer was rinsed with PBS (0.1 M, pH 7.4), saturated with 1% (w/v) bovine serum albumin (BSA) for 1 h at 37 °C, and incubated overnight with monoclonal antibody against human osteocalcin (Takara) diluted at 1/500 in PBS (0.1 M, pH 7.4). Fixed immunoglobulins were detected with a peroxidase-labeled secondary antibody against mouse immunoglobulins and a detection kit (DAKO).

Cytotoxicity assay: HBMSCs were plated at 5×10^3 cells cm^{-2} into 36-well plates in IMDM supplemented with 10% (v/v) FCS, and grown to confluency.[10] Confluent cultures were incubated for 24 hours in IMDM containing 10% (v/v) FCS in the presence of different concentrations of compounds **3a**, **3b**, **5a**, **5b**, **6**, **11**, **13**, **14**, **15**, **17**, **18**, **20**, **23**, and **25** (1 mM, 0.5 mM, 0.1 mM, and 0.01 mM) in PBS buffer (0.1 M, pH 7.4) containing 10 mM $MgCl_2$ and 0.1% (w/v) BSA. Two control extracts were used: a positive control with phenol at 64 g L^{-1}, which induces a reproducible cytotoxic response, and a negative control with the culture medium, IMDM. At the end of the incubation time, the MTT assay was performed by a previously described procedure.[11]

Hypoxanthine active transport: Nonconfluent HBMSC cultures plated into 24-well plates were mixed with various concentrations (1, 10, 50, 150, and 300 µM) of [^{14}C]hypoxanthine in PBS buffer. The [^{14}C]hypoxanthine uptake into the cells was measured at different times (15, 30, 60, 90, 120, and 150 min). After each time interval, the transport medium was removed, and the wells were rinsed three times with the PBS buffer to remove nonincorporated [^{14}C]hypoxanthine. Cells were lysed by addition of water (500 µL). Lysates were collected and radioactivity was counted by liquid scintillation in a Packard β counter. The same experiment was conducted with cell cultures that had previously been incubated with CCCP (200 µM) and NaN_3 (1 mM) in PBS buffer to block the energy sources within the cells. All data are the results of duplicate experiments.

Inhibition of hypoxanthine transport: Noncytotoxic compounds **5a, 6, 13, 14, 15, 17**, and **25** (100 µM in PBS buffer) and [^{14}C]hypoxanthine (1 µM) were added simultaneously to triplicate monolayer wells of nonconfluent HBMSC cultures. The [^{14}C]hypoxanthine uptake into the cells was measured at different intervals (15, 30, 60, 90, 120, and 150 min). After each time interval, the cells were treated as described above.

By the above procedure, cells were incubated with varying concentrations of analogues **13** and **14** (10 µM, 100 µM, 150 µM, and 250 µM in PBS buffer) in the presence of [^{14}C]hypoxanthine at an approximate K_m concentration of 2 µM.

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