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Synthesis and evaluation of in vitro anticancer activity of some novel isopentenyladenosine derivatives

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ABSTRACT

The present study describes the synthesis, the characterization and the evaluation of some derivatives of N^6-isopentenyladenosine on T24 human bladder carcinoma cells. In particular we have modified the hydroxyl groups in the ribose moiety, the position of the isopentenyl chain in the purine ring and the base moiety. The structures of the compounds were confirmed by standard studies of NMR, MS and elemental analysis. We here show that only two derivatives, 1-(3-methyl-2-butenylamino)-9-(3′-deoxy-β-D-ribofuranosyl)-purine hydrobromide and 2-amino-6-(3-methyl-2- butenylamino)-9-(β-D-ribofuranosyl)-purine, inhibit the growth of T24 cells, although to a lower extent than N^6-isopentenyladenosine. We conclude that the integrity of ribosidic and purine moiety and the N^6 position of the chain are essential for maintaining the antiproliferative activity.

1. Introduction

N^6-Isopentenyladenosine (iPA, **1a**) is a naturally occurring nucleoside present in yeast and mammalian t-RNA.[1,2] In vitro iPA (**1a**) was found to inhibit cell multiplication on a variety of human epithelial cancers.[3] Recently, iPA has been shown to act by inhibiting DNA synthesis, causing a cell cycle arrest that correlates with a decrease in the levels of cyclin E, cyclin A and cyclin D1 and with a concomitant increase in the levels of cyclin-dependent kinase inhibitor and p27kip1. Moreover, iPA induces apoptosis through the downregulation of antiapoptotic products and caspase-3 activation.[4]

However, in vivo testing of iPA in the conventional animal tumour screening revealed a modest antitumoral activity which might be due to the short plasma half-life of iPA, in analogy to other nucleosides.[5,6]

To continue our studies concerning the relation between structure and biological activity,[7] as well as to obtain compounds endowed with antiproliferative activity both in vitro and in vivo, we have synthesized a number of iPA analogues modified in the sugar portion (compounds **1b**–**f**), in the alkylation position (compounds **2b**–**f**), in the purine base (compounds **3a**–**c**) or with a stable base-sugar linkage (compounds **4a**–**b**). We have studied these molecules for their antiproliferative activity on T24 cells, a cell line established from a human urinary bladder cancer patient. Bladder cancer is the fourth most common cancer among men and the ninth most common cancer among women.[8] Its treatment remains a challenge despite significant improvements in preventing disease progression and improving survival, because bladder cancer is characterized by a high rate of tumour recurrence and potential progression, regardless of treatment with surgery, chemotherapy, or immunotherapy. We have previously demonstrated that iPA inhibited the proliferation of human bladder cancer cells in a dose-dependent fashion.[7]

One of our goals is to individuate structural modifications necessary to obtain iPA analogues which maintain biological activity in vivo. Indeed, we recently reported the synthesis of iPA analogues with modifications at N^9-position which were investigated for their biological activity in vitro.[7] We here report the synthesis of iPA derivatives modified on ribose and base moiety evaluated for their biological activity on bladder carcinoma cells.

2. Results and discussion

2.1. Chemistry

To investigate the structure-anticancer in vitro activity relationship of the lead compound iPA (**1a**) we modified the sugar portion (compounds **1b**–**f**), the alkylation position (compounds **2b**–**f**) and the purine base (compounds **3a**–**c**) (Fig. 1).

Since our aim is to maintain the antiproliferative activity of iPA in vivo, we were also interested in synthesizing compounds resistant to the action of purine nucleoside phosphorylase (PNP), one of

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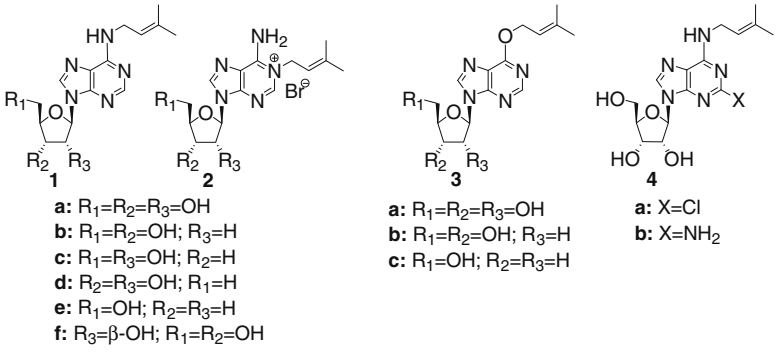

Figure 1. Structures of the modified iPA analogues.

the key enzymes in the purine-metabolism.[9] Since the substitution of the hydrogen atom at the 2-position of the purine ring renders the molecules resistant to metabolic enzymes[10] we have also synthesized compounds **4a**–**b**.

Initially, it was necessary to obtain the key intermediates 3′-deoxyadenosine, 5′-deoxyadenosine, and 2′,3′-dideoxyadenosine that were synthesized according to the procedures reported in the literature[11–13] and were identified by their physical and spectroscopic data. On the contrary, the intermediates adenine-9-β-D-arabinofuranoside, adenine-2-chloro-9-β-D-ribofuranoside and adenine-2-amino-9-β-D-ribofuranoside were commercially available.

All monosubstituted N^6-isopentenyl-deoxyadenosine derivatives **1b**–**e**, were prepared by N^1-alkylation of the opportune substrate with 3,3-dimethylallylbromide. The N^1-alkylated derivatives **2b**–**e** were submitted to Dimroth rearrangement to N^6-isomers.[14,15] We observed that this alkylation-rearrangement procedure invariably contained a small amount of the starting nucleoside that was removed by column chromatography. Analogous reactions using adenine 9-β-D-arabinofuranoside gave at first the N^1-alkylated derivative **2f** and then the corresponding N^6-isopentenyl derivative **1f**.

2′,3′-Dideoxyinosine and 2′-deoxyinosine, key intermediates to obtain compounds **3b** and **3c**, respectively, were obtained by enzymatic deamination of 2′-deoxyadenosine and 2′,3′-dideoxyadenosine with adenosine deaminase.[16] The one step alkylation reaction gave the desired products **3b** and **3c**. Isopentenyl-derivatives **3a**, **4a** and **4b** were obtained by one step alkylation reaction which yield only N^6-alkylated compounds starting from the commercially available inosine-9-β-D-ribofuranoside, adenine-2-chloro-9-β-D-ribofuranoside and adenine-2-amino-9-β-D-ribofuranoside.

The structure of the synthetic intermediates and of the derivatives **1b**–**f**, **2b**–**f**, **3a**–**c** and **4a**–**b** was confirmed from MS, 1D and 2D NMR spectroscopic data and elemental (C, H, N) analyses which allowed the correct identification and determined the purity of all compounds (see Section 5).

The correct site of N-alkylation was elucidated by NOE experiments. NOE correlation between H–N^6 and α-CH_2 and β-CH of the isopentenyl moiety could be detected in N^6-alkylated derivatives **1b**–**f,** while it is absent in N^1-alkylated derivatives **2b**–**f**, thus demonstrating the binding of the isopentenyl chain in N^6-position.

Moreover α-CH_2 in side chain resonances of N^6-alkylated compounds **1b**–**f**, is more shielded than α-CH_2 of N^1-alkylated compounds **2b**–**f**.

2.2. Biological assays

On the basis of results obtained from dose/response curve performed with iPA,[7] we utilized 10 μM of iPA and its analogues **1b**–**f**, **2b**–**f**, **3a**–**c** and **4a**–**b** to compare their capability to inhibit cell proliferation and clonogenicity.

Initially, the anti-proliferative capacity of the different compounds was assessed on quiescent and proliferating T24 cells by the microtiter tetrazolium assay (MTT) as described in the methods. Our results show the anti-proliferative activity of iPA both in quiescent (Fig. 2a) and proliferating (Fig. 2b) T24 cells and the lack of any effect of its derivatives **1b**, **1d**, **1f**, **2b**, **2e**, **2f**, **3a**–**c** and **4a**.

Among all synthesized iPA derivatives, compound **4b** showed a slight, albeit statistically significant, inhibitory effect on proliferating cells. Interestingly, **4b** significantly reduced quiescent cell number, probably because of a cytotoxic effect as previously described for iPA.[7] It is noteworthy that, when added to the medium of quiescent T24 cells, compound **2c** showed a selective capacity to reduce cell number, while it did not affect the growth of the cells in 10% FBS. We found that 3′-deoxy-adenosine had no effects on T24 cells in the two culture conditions tested (data not shown).

The molecules that had shown some inhibitory effects, namely **2c** and **4b,** were further investigated by proliferation assays both

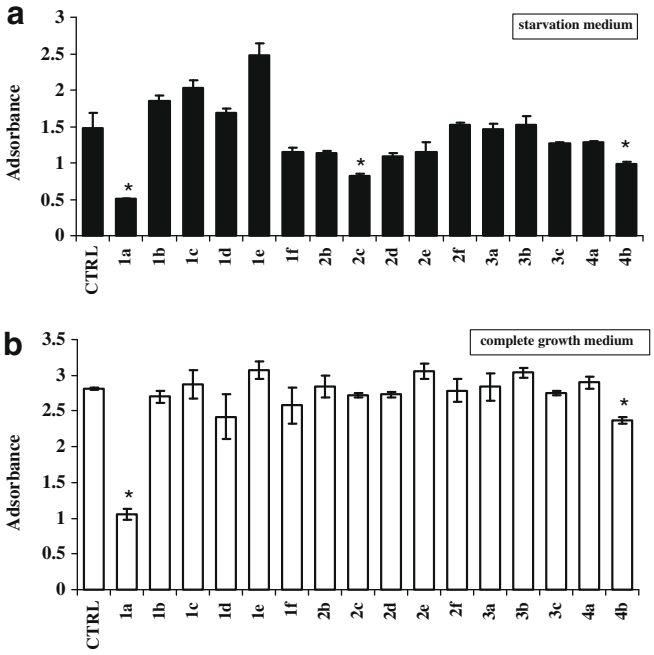

Figure 2. MTT assay on T24 cells treated with iPA and derivatives. (a) MTT quantification on T24 cells exposed to starvation medium and treated with 10 μM of iPA or synthesized derivatives. (b) MTT quantification on T24 cells exposed to complete growth medium and treated with 10 μM of iPA or synthesized derivatives. * $p < 0.05$.

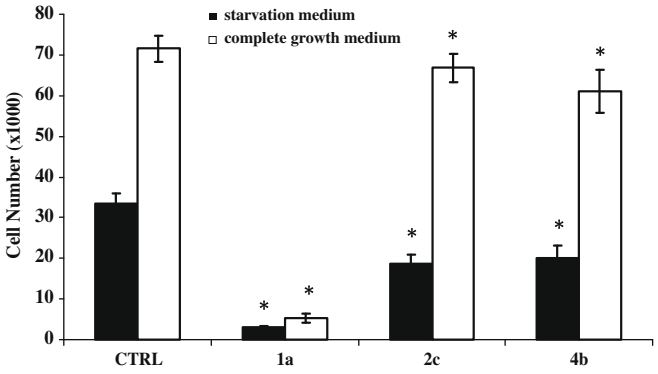

Figure 3. Proliferation assay on T24 cells treated with iPA and derivatives. The cells were treated with 10 μM iPA or selected derivatives and counted after 72 h of treatment. * p <0.05.

on quiescent and proliferating T24 cells. Figure 3 shows counted cells after 72 h of treatment, only **4b** had a slight inhibitory effect on cell cultured in complete growth medium while compounds **2c** and **4b** markedly reduced the number of quiescent cells.

We then performed clonogenic assays in the presence of iPA and all its derivatives (10 μM). After a week, colonies were fixed with crystal violet and the plates were photographed. We confirmed that iPA inhibited the formation of clones, while, in agreement with the aforementioned results, apart from a slight reduction of number and dimension of clones in cells treated with **4b**, all the compounds did not exert any effect on the formation of clones (Fig. 4).

We also evaluated the effects of iPA and synthesized analogues on cell migration, which is fundamental for invasion and metastasis. T24 cells were treated for 8 h with iPA or analogues and then exposed to EGF (10 ng/mL) for additional 14 h. iPA and its derivatives exerted no effect on basal or EGF-stimulated migration in T24 cells. Figure 5 shows that, in spite of their antiproliferative activity, iPA, compound **2c** and **4b** do not inhibit cell migration.

3. Conclusion

In summary, we have synthesized a series of novel iPA derivatives and we have determined their antiproliferative activity after modification of the hydroxyl groups in the ribose moiety, of the alkylation position and of the base moiety.

All the modifications drastically inhibited iPA biological activity. On these bases, we can conclude that the iPA ribofuranosidic and base moiety are pivotal for iPA antiproliferative activity in vitro. Among all the synthesized derivatives only compound **4b**, characterized by the presence of an amino group in position 2, showed a slight inhibitory effect on proliferating cells and a more evident action on quiescent cells. Therefore, **4b** is the only compound which mimics iPA, even though with a lower efficiency. We hypothesize that the presence of the amino group in 2-position in **4b** renders this molecule more bioavailable than the others. Interestingly, compound **2c**, which has the isopentenyl chain in N^1-position, selectively acts on quiescent cells. The differential action of compound **2c** on quiescent and proliferating cells is puzzling. That a molecule selectively acts on quiescent cells might be relevant in primary or metastatic tumors, where part of the cells are dormient and might reactivate after many years, as often reported in the case of bladder carcinoma. However, at the moment we can not provide any explanation for these different effects. More studies are necessary to understand the mechanisms involved.

It is noteworthy that iPA and its derivatives exert no effect on cell migration, which is fundamental in invasion and metastasis.

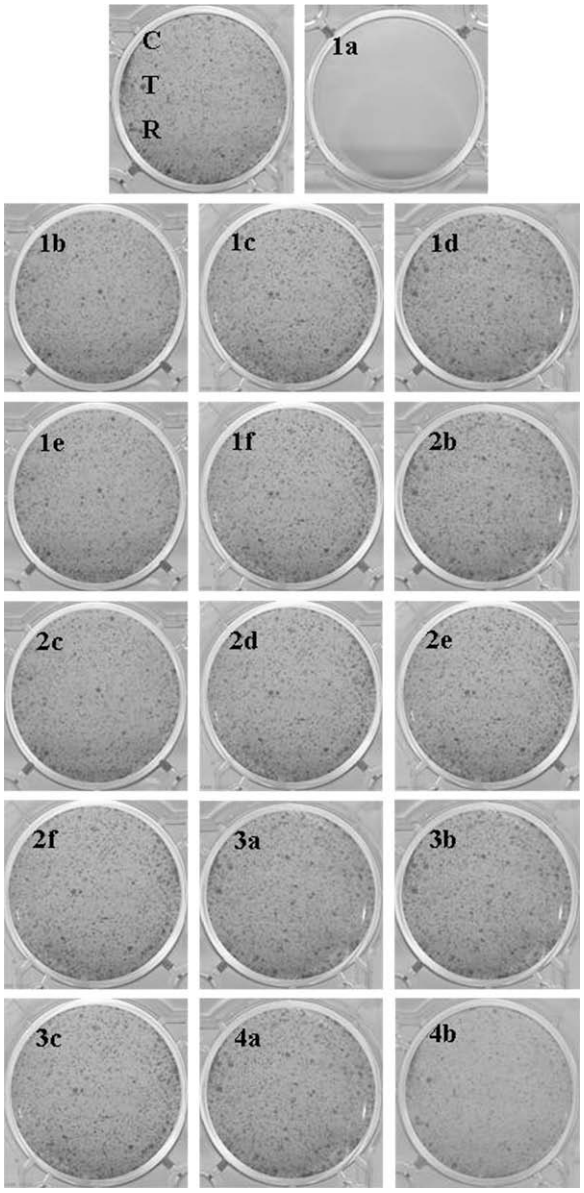

Figure 4. Clonogenic assay on T24 cells treated with iPA and derivatives. T24 cells were seeded at very low density, treated with iPA and derivatives for 72 h and maintained thereafter in culture medium alone until the end of the experiment. The cells were stained and photographed (magnification 40×).

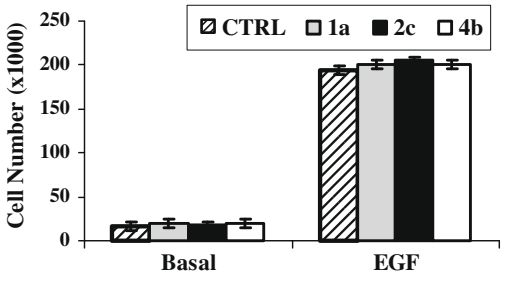

Figure 5. Effects of iPA, **2c** and **4b** on T24 bladder cancer cell migration. The cells were treated with EGF (100 ng/ml) and the different molecules (or an equal amount of DMSO as a control—CTRL-) for 16 h. The wound area was calculated by a specific software and expressed using an arbitrary value scale.

We argue that iPA and its derivatives do not interfere with the complex signalling pathways necessary for cell migration.

In conclusion, our results demonstrate that the integrity of ribosidic and purine moiety and the N^6-position of the isopentenyl chain are essential for maintaining the antiproliferative activity. Indeed, it is sufficient to modify the stereochemistry of 2′-hydroxy group in the ribose moiety (compound **1f**) or to substitute a nitrogen with an oxygen atom in the 6-position of the base (compound **3a**) to completely abolish the activity.

The results of this investigation prompt us to continue the development and testing of novel derivatives of iPA with the aim of individuating novel biologically active compounds which maintain their activity also in vivo.

4. Experimental

4.1. General

Melting points were determined with a Stuart Scientific SMP3 melting point apparatus and left uncorrected. Optical rotations were measured on a Perkin–Elmer 241 polarimeter (sodium D line at 25 °C). NMR spectra were done on a Bruker AVANCE 500 spectrometer equipped with a 5 mm broadband reverse probe with field *z*-gradient operating at 500.13 MHz for ^{1}H. All NMR spectra were recorded at 298 K in $CDCl_3$ (isotopic enrichment 99.95%) or CD_3OD (isotopic enrichment 99.95%) solution and the chemical shifts were reported on a δ (ppm) scale. The central peak of $CDCl_3$ signals (7.26 ppm) and of CD_3OD signals (3.31 ppm) were used as internal reference standard. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, and br, broad singlet.

Mass spectra were recorded on a Finnigan LCQdeca (ThermoQuest) in ESI negative ion mode, kV 5.00, 220 °C, 15 V. Only significant *m/z* peaks, with their percentage of relative intensity in parentheses are reported.

Reactions progress was monitored by analytical thin-layer chromatography (TLC) on pre-coated glass plates (Silica Gel 60 F254-plate-Merck, Darmstaat, Germany) and the products were visualized by UV light. Elemental analyses were obtained for all intermediates and are within ±0.4% of theoretical values.

Adenosine, 2′-deoxyadenosine, inosine, adenine 9-β-D-arabinofuranoside, adenine-2-chloro-9-β-D-ribofuranoside and adenine-2-amino 9-β-D-ribofuranoside, the other reagents and all solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Organic solvents were dried in the presence of appropriate drying agents and were stored over suitable molecular sieves.

4.2. General procedure for N^6-alkylation

$BaCO_3$ (2.4 mmol) and 3,3-dimethylallylbromide (2.25 mmol) were added to a solution of nucleoside derivative (1.5 mmol) in DMF (20 mL). The mixture was stirred at room temperature for 24 h while protected from light and moisture. TLC indicated that N^1-alkylation was about 90% complete. The moisture was filtered using a Celite pad and washed with DMF. The combined filtrate was evaporated to a small volume and purified by column chromatography on silica gel. The N^1-alkylated derivative was treated with Me_2NH–MeOH (1 M, 4.5 mL) at room temperature for 16 h. The solvent was removed and the residue purified by column chromatography on silica gel.

4.2.1. 6-(3-Methyl-2-butenylamino)-9-(2′-deoxy-β-D-ribofuranosyl)-purine (1b)

4.2.1.1. 1-(3-Methyl-2-butenylamino)-9-(2′-deoxy-β-D-ribofuranosyl)-purine hydrobromide (2b). White solid; 62% yield; R_f = 0.25 (CH_2Cl_2/MeOH, 90:10); mp 104–106 °C; $[\alpha]_D^{20}$ −19.8 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.86 (s, 3H, CH_3), 1.88 (s, 3H, CH_3), 2.51 (ddd, *J* = 3.5 Hz, *J* = 6.3 Hz, *J* = 13.2 Hz, 1H, H2′a), 2.77 (ddd, *J* = 6.3 Hz, *J* = 6.3 Hz, *J* = 13.2 Hz, 1H, H2′b), 3.76 (dd, *J* = 4.2 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.82 (dd, *J* = 3.5 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.07 (ddd, *J* = 2.8 Hz, *J* = 3.5 Hz, *J* = 4.2 Hz, 1H, H4′), 4.59 (ddd, *J* = 2.8 Hz, *J* = 3.5 Hz, *J* = 6.3 Hz, 1H, H3′), 4.87 (d, *J* = 6.6 Hz, 2H, α-CH_2), 5.44 (t, *J* = 6.6 Hz, 1H, β-CH), 6.48 (dd, *J* = 6.3 Hz, *J* = 6.3 Hz, 1H, H1′), 8.40 (s, 1H, H2), 8.53 (s, 1H, H8); MS (EI): *m/z* 320 (M−HBr+1, 100%). Anal. Calcd for $C_{15}H_{22}BrN_5O_3$: C, 45.01; H, 5.54; Br, 19.96; N, 17.50; O, 11.99. Found: C, 45.12; H, 5.44; Br, 19.82; N, 17.41.

4.2.1.2. 6-(3-Methyl-2-butenylamino)-9-(2′-deoxy-β-D-ribofuranosyl)-purine (1b). White solid; 86% yield; R_f = 0.63 (CH_2Cl_2/MeOH, 90:10); mp 108–109 °C; $[\alpha]_D^{20}$ −19.2 (*c* 1, MeOH) {lit.[17] mp 106–109 °C; $[\alpha]_D^{20}$ −19.5 (*c* 1, MeOH)}; ^{1}H NMR (CD_3OD) δ = 1.77 (s, 6H, 2 × CH_3), 2.42 (ddd, *J* = 3.5 Hz, *J* = 8.2 Hz, *J* = 13.4 Hz, 1H, H2′a), 2.82 (ddd, *J* = 5.9 Hz, *J* = 6.3 Hz, *J* = 13.4 Hz, 1H, H2′b), 3.76 (dd, *J* = 3.1 Hz, *J* = 12.3 Hz, 1H, H5′a), 3.86 (dd, *J* = 2.8 Hz, *J* = 12.3 Hz, 1H, H5′b), 4.09 (ddd, *J* = 2.1 Hz, *J* = 2.8 Hz, *J* = 3.1 Hz, 1H, H4′), 4.17 (br, 2H, α-CH_2), 4.59 (ddd, *J* = 2.1 Hz, *J* = 3.5 Hz, *J* = 5.9 Hz, 1H, H3′), 5.39 (t, *J* = 6.9 Hz, 1H, β-CH), 6.43 (dd, *J* = 6.3 Hz, *J* = 8.2 Hz, 1H, H1′), 8.22 (s, 1H, H2), 8.26 (s, 1H, H8); MS (EI): *m/z* 320 (M+1, 100%), 343 (M+23, 32%).

4.2.2. 6-(3-Methyl-2-butenylamino)-9-(3′-deoxy-β-D-ribofuranosyl)-purine (1c)

4.2.2.1. 1-(3-Methyl-2-butenylamino)-9-(3′-deoxy-β-D-ribofuranosyl)-purine hydrobromide (2c). White solid; 55% yield; R_f = 0.28 (CH_2Cl_2/MeOH, 90:10); mp 177–179 °C; $[\alpha]_D^{20}$ −44.7 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.86 (s, 3H, CH_3), 1.88 (s, 3H, CH_3), 2.05 (ddd, *J* = 2.7 Hz, *J* = 6.2 Hz, Hz, *J* = 13.8 Hz, 1H, H3′a), 2.36 (ddd, *J* = 6.2 Hz, *J* = 9.7, *J* = 13.8 Hz, 1H, H3′b), 3.71 (dd, *J* = 3.4 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.94 (dd, *J* = 2.8 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.57 (dddd, *J* = 2.8 Hz, *J* = 3.4 Hz, *J* = 6.2 Hz, *J* = 9.7 Hz, 1H, H4′), 4.59 (ddd, *J* = 1.4 Hz, *J* = 2.7 Hz, *J* = 6.2 Hz, 1H, H2′), 4.87 (d, *J* = 6.6 Hz, 2H, α-CH_2), 5.44 (t, *J* = 6.6 Hz, 1H, β-CH), 6.04 (d, *J* = 1.4 Hz, 1H, H1′), 8.37 (s, 1H, H2), 8.60 (s, 1H, H8); MS (EI): *m/z* 320 (M−HBr+1, 100%), 343 (M−HBr+1+(23), 80%). Anal. Calcd for $C_{15}H_{22}BrN_5O_3$: C, 45.01; H, 5.54; Br, 19.96; N, 17.50; O, 11.99. Found: C, 45.15; H, 5.48; Br, 20.02; N, 17.60.

4.2.2.2. 6-(3-Methyl-2-butenylamino)-9-(3′-deoxy-β-D-ribofuranosyl)-purine (1c). White solid; 88% yield; R_f = 0.78 (CH_2Cl_2/MeOH, 90:10); mp 148–149 °C; $[\alpha]_D^{20}$ −42.8 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.79 (s, 6H, 2 × CH_3), 2.09 (ddd, *J* = 3.8 Hz, *J* = 6.6 Hz, *J* = 13.2 Hz, 1H, H3′a), 2.39 (ddd, *J* = 6.2 Hz, *J* = 9.4 Hz, *J* = 13.2 Hz, 1H, H3′b), 3.68 (dd, *J* = 3.5 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.94 (dd, *J* = 2.4 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.19 (br, 2H, α-CH_2), 4.54 (dddd, *J* = 2.4 Hz, *J* = 3.5 Hz, *J* = 6.6 Hz, *J* = 9.4 Hz, 1H, H4′), 4.72 (ddd, *J* = 2.8 Hz, *J* = 3.8 Hz, *J* = 6.2 Hz, 1H, H2′), 5.41 (t, *J* = 6.9 Hz, 1H, β-CH), 5.96 (d, *J* = 2.8 Hz, 1H, H1′), 8.26 (s, 1H, H2), 8.37 (s, 1H, H8); MS (EI): *m/z* 320 (M+1, 20%), 389 (M+1+(3 × 23), 100%). Anal. Calcd for $C_{15}H_{21}N_5O_3$: C, 56.41; H, 6.63; N, 21.93; O, 15.03. Found C, 56.28; H, 6.86; N, 21.79.

4.2.3. 6-(3-Methyl-2-butenylamino)-9-(5′-deoxy-β-D-ribofuranosyl)-purine (1d)

4.2.3.1. 1-(3-Methyl-2-butenylamino)-9-(5′-deoxy-β-D-ribofuranosyl)-purine hydrobromide (2d). White solid; 48% yield; R_f = 0.36 (CH_2Cl_2/MeOH, 90:10); mp 96–98 °C; $[\alpha]_D^{20}$ −23.2 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 41.45 (d, *J* = 6.5 Hz, 3H, H5′) 1.83 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 4.09 (dd, *J* = 4.5 Hz, *J* = 5.2 Hz, 1H, H3′), 4.16 (dq, *J* = 5.2 Hz, *J* = 6.5 Hz, 1H, H4′), 4.68 (dd, *J* = 4.5, *J* = 5.2, 1H, H2′), 4.90 (d, *J* = 6.5 Hz, 2H, α-CH_2), 5.40 (t, *J* = 6.5 Hz, 1H, β-CH), 6.04 (d, *J* = 4.5 Hz, 1H, H1′), 8.46 (s, 1H, H2), 8.51 (s,

1H, H8); MS (EI): *m/z* 343 (M−HBr+1+23, 100%). Anal. Calcd for $C_{15}H_{22}BrN_5O_3$: C, 45.01; H, 5.54; Br, 19.96; N, 17.50; O, 11.99. Found: C, 44.98; H, 5.39; N, 17.61.

4.2.3.2. 6-(3-Methyl-2-butenylamino)-9-(5′-deoxy-β-D-ribofuranosyl)-purine (1d). White solid; 77% yield; R_f = 0.67 (CH_2Cl_2/MeOH, 90:10); mp 124–126 °C {lit.[15] mp 96–97 °C}; $[\alpha]_D^{20}$ −37.7; ^{1}H NMR (CD_3OD) δ = 1.45 (d, *J* = 6.5 Hz, 3H, CH_3), 1.79 (s, 6H, 2 × CH_3), 4.09 (dd, *J* = 4.5 Hz, *J* = 5.2 Hz, 1H, H3′), 4.14 (dq, *J* = 5.2 Hz, *J* = 6.5 Hz, 1H, H4′), 4.20 (br, 2H, α-CH_2), 4.70 (dd, *J* = 4.5 Hz, *J* = 5.2 Hz, 1H, H2′), 5.41 (t, *J* = 6.9 Hz, 1H, β-CH), 5.97 (d, *J* = 4.5 Hz, 1H, H1′), 8.20 (s, 1H, H8), 8.27 (s, 1H, H2); MS (EI): *m/z* 388 (M+(3 × 23), 100%).

4.2.4. 6-(3-Methyl-2-butenylamino)-9-(2′,3′-dideoxy-β-D-ribofuranosyl)-purine (1e)

4.2.4.1. 1-(3-Methyl-2-butenylamino)-9-(2′,3′-dideoxy-β-D-ribofuranosyl)-purine hydrobromide (2e). White solid; 45% yield; R_f = 0.22 (CH_2Cl_2/MeOH, 90:10); mp 100–102 °C; $[\alpha]_D^{20}$ −5.73 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.84 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 2.12–2.24 (m, 2H, H3′), 2.48–2.56 (m, 2H, H2′), 3.69 (dd, *J* = 4.5 Hz, *J* = 12.1 Hz, 1H, H5′a), 3.86 (dd, *J* = 3.1 Hz, *J* = 12.1 Hz, 1H, H5′b), 4.26–4.30 (m, 1H, H4′), 4.81 (d, *J* = 6.9 Hz, 2H, α-CH_2), 5.43 (t, *J* = 6.9 Hz, 1H, β-CH), 6.32 (dd, *J* = 6.6 Hz, *J* = 3.1 Hz, 1H, H1′), 8.27 (s, 1H, H2), 8.49 (s, 1H, H8); MS (EI): *m/z* 304 (M−HBr+1, 100%). Anal. Calcd for $C_{15}H_{22}BrN_5O_2$: C, 46.88; H, 5.77; Br, 20.79; N, 18.23; O, 8.33. Found: C, 46.72; H, 5.70; Br, 20.82; N, 18.38.

4.2.4.2. 6-(3-Methyl-2-butenylamino)-9-(2′,3′-dideoxy-β-D-ribofuranosyl)-purine (1e). White solid; 82% yield; R_f = 0.74 (CH_2Cl_2/MeOH, 90:10); mp 105–107 °C; $[\alpha]_D^{20}$ −12.6 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.79 (s, 6H, 2 × CH_3), 2.12–2.24 (m, 2H, H3′), 2.48–2.58 (m, 2H, H2′), 3.68 (dd, *J* = 3.8 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.89 (dd, *J* = 2.8 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.19 (br, 2H, α-CH_2), 4.26–4.31 (m, 1H, H4′), 5.40 (t, *J* = 6.9 Hz, 1H, β-CH), 6.30 (dd, *J* = 4.5 Hz, *J* = 5.9 Hz, 1H, H1′), 8.25 (s, 1H, H2), 8.36 (s, 1H, H8); MS (EI): *m/z* 304 (M+1, 100%). Anal. Calcd for $C_{15}H_{21}N_5O_2$: C, 59.39; H, 6.98; N, 23.09; O, 10.55. Found C, 59.26; H, 6.88; N, 23.14.

4.2.5. 6-(3-Methyl-2-butenylamino)-9-β-D-arabinofuranosyl)-purine (1f)

4.2.5.1. 1-(3-Methyl-2-butenylamino)-9-β-D-arabinofuranosyl)-purine hydrobromide (2f). White solid; 52% yield; R_f = 0.28 (CH_2Cl_2/MeOH, 90:10); mp 147–148; $[\alpha]_D^{20}$ +2.2 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.87 (s, 3H, CH_3), 1.90 (s, 3H, CH_3), 3.87 (dd, *J* = 3.4 Hz, *J* = 11.9 Hz, 1H, H5′a), 3.91 (dd, *J* = 4.5 Hz, *J* = 11.9 Hz, 1H, H5′b), 4.02 (ddd, *J* = 3.4 Hz, *J* = 4.5 Hz, *J* = 4.5 Hz, 1H, H4′), 4.31 (dd, *J* = 4.5 Hz, *J* = 4.5 Hz, 1H, H3′), 4.35 (dd, *J* = 4.5 Hz, *J* = 4.9 Hz, 1H, H2′), 4.96 (d, *J* = 6.8 Hz, 2H, α-CH_2), 5.45 (tt, *J* = 1.5 Hz, *J* = 6.8 Hz, 1H, β-CH), 6.46 (d, *J* = 4.9 Hz, 1H, H1′), 8.55 (s, 1H, H2), 8.26 (s, 1H, H8); MS (EI) *m/z* 336 (M−HBr+1, 100%). Anal. Calcd for $C_{15}H_{22}BrN_5O_4$: C, 43.28; H, 5.33; Br, 19.20; N, 16.82; O, 15.37. Found: C, 43.12; H, 5.44; Br, 19.12; N, 16.91.

4.2.5.2. 6-(3-Methyl-2-butenylamino)-9-β-D-arabinofuranosyl)-purine (1f). White solid; 84% yield; R_f = 0.65 (CH_2Cl_2/MeOH, 90:10); mp 180–182 °C; $[\alpha]_D^{20}$ 2.5 (*c* 1, MeOH) {lit.[15] mp 161.5–162 °C; $[\alpha]_D^{20}$ 1.9 (*c* 1, MeOH)}; ^{1}H NMR (CD_3OD) δ = 1.79 (s, 6H, 2 × CH_3), 3.86 (dd, *J* = 4.5 Hz, *J* = 11.9 Hz, 1H, H5′a), 3.92 (dd, *J* = 3.5 Hz, *J* = 11.9 Hz, 1H, H5′b), 4.00 (ddd, *J* = 3.5 Hz, *J* = 4.5 Hz, *J* = 4.9 Hz, 1H, H4′), 4.20 (d, *J* = 6.9 Hz, 2H, α-CH_2), 4.28–4.32 (m, 2H, H2′, H3′), 5.41 (t, *J* = 6.9 Hz, 1H, β-CH), 6.40 (d, *J* = 4.9 Hz, 1H, H1′), 8.25 (s, 1H, H2), 8.32 (s, 1H, H8); MS (EI): *m/z* 320 (M+1, 100%), 343 (M+23, 52%).

4.2.6. 6-(3-Methyl-2-butenyloxy)-9-(β-D-ribofuranosyl)-purine (3a)

White solid; 46% yield; R_f = 0.37 (CH_2Cl_2/MeOH, 95:5); mp 56–58 °C; $[\alpha]_D^{20}$ −37.4 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.79 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 3.77 (dd, *J* = 3.1 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.88 (dd, *J* = 2.8 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.15 (ddd, *J* = 2.8 Hz, *J* = 3.1 Hz, *J* = 3.8 Hz, 1H, H4′), 4.34 (dd, *J* = 3.8 Hz, *J* = 5.6 Hz, 1H, H3′), 4.64 (dd, *J* = 5.6 Hz, *J* = 5.6 Hz, 1H, H2′), 4.72 (d, *J* = 6.9 Hz, 2H, α-CH_2), 5.36 (t, *J* = 6.9 Hz, 1H, β-CH), 6.02 (d, *J* = 5.6 Hz, 1H, H1′), 8.29 (s, 1H, H2), 8.33 (s, 1H, H8); MS (EI): *m/z* 359 (M+23, 47%), 695 (2M+23, 100%). Anal. Calcd for $C_{15}H_{20}N_4O_5$: C, 53.56; H, 5.99; N, 16.66; O, 23.78. Found C, 53.42; H, 5.88; N, 16.49.

4.2.7. 6-(3-Methyl-2-butenyloxy)-9-(2′-deoxy-β-D-ribofuranosyl)-purine (3b)

White solid; 52% yield; R_f = 0.21 (CH_2Cl_2/MeOH, 90:10); mp 126–128 °C; $[\alpha]_D^{20}$ −4.0 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.79 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 2.46 (ddd, *J* = 3.2 Hz, *J* = 6.3 Hz, *J* = 13.2 Hz, 1H, H2′a), 2.76 (ddd, *J* = 6.3 Hz, *J* = 6.3 Hz, *J* = 13.2 Hz, 1H, H2′b), 3.75 (dd, *J* = 3.5 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.82 (dd, *J* = 3.5 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.05 (ddd, *J* = 3.5 Hz, *J* = 3.5 Hz, *J* = 3.5 Hz, 1H, H4′), 4.58 (ddd, *J* = 3.2 Hz, *J* = 3.5 Hz, *J* = 6.3 Hz, 1H, H3′), 4.72 (d, *J* = 6.6 Hz, 2H, α-CH_2), 5.38 (t, *J* = 6.6 Hz, 1H, β-CH), 6.44 (dd, *J* = 6.3 Hz, *J* = 6.3 Hz, 1H, H1′), 8.28 (s, 1H, H2), 8.32 (s, 1H, H8); MS (EI): *m/z* 344 (M+1+23, 28%), 665 (2 × (M+1)+23, 100%). Anal. Calcd for $C_{15}H_{20}N_4O_4$: C: 56.24, H: 6.29, N: 17.49, O: 19.98. Found C, 56.12; H, 6.10; N, 17.39.

4.2.8. 6-(3-Methyl-2-butenyloxy)-9-(2′,3′-deoxy-β-D-ribofuranosyl)-purine (3c)

White solid; 68% yield; R_f = 0.53 (CH_2Cl_2/MeOH, 90:10); mp 110–112 °C; $[\alpha]_D^{20}$ −4.08 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.79 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 2.07–2.20 (m, 2H, H3′), 2.47–2.61 (m, 2H, H2′), 3.69 (dd, *J* = 4.5 Hz, *J* = 11.8 Hz, 1H, H5′a), 3.85 (dd, *J* = 3.2 Hz, *J* = 11.8 Hz, 1H, H5′b), 4.24–4.29 (m, 1H, H4′), 4.72 (d, *J* = 6.9 Hz, 2H, α-CH_2), 5.36 (t, *J* = 6.9 Hz, 1H, β-CH), 6.31 (dd, *J* = 3.5 Hz, *J* = 6.3 Hz, 1H, H1′), 8.24 (s, 1H, H2), 8.27 (s, 1H, H8); MS (EI): *m/z* 336 (M−1, 30%), 673 (2M−1, 100%). Anal. Calcd for $C_{15}H_{20}N_4O_3$: C, 59.20; H, 6.62; N, 18.41; O, 15.77. Found C, 59.12; H, 6.48; N, 18.26.

4.2.9. 2-Chloro-6-(3-methyl-2- butenylamino)-9-(β-D-ribofuranosyl)-purine (4a)

White solid; 34% yield; R_f = 0.27 (CH_2Cl_2/MeOH, 95:5); mp 148–150 °C; $[\alpha]_D^{20}$ −6.9 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.74 (s, 6H, 2 × CH_3), 3.75 (dd, *J* = 3.1 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.86 (dd, *J* = 2.4 Hz, *J* = 12.2 Hz, 1H, H5′b), 3.95 (d, *J* = 7.0 Hz, 2H, α-CH_2), 4.14 (ddd, *J* = 2.4 Hz, *J* = 3.1 Hz, *J* = 3.1 Hz, 1H, H4′), 4.33 (dd, *J* = 3.1 Hz, *J* = 5.4 Hz, 1H, H3′), 4.79 (dd, *J* = 5.4 Hz, *J* = 6.7 Hz, 1H, H2′), 5.33 (t, *J* = 7.0 Hz, 1H, β-CH), 5.85 (d, *J* = 6.7 Hz, 1H, H1′), 8.00 (s, 1H, H8); MS (EI): *m/z* 370 (M+1, 100%). Anal. Calcd for $C_{15}H_{20}ClN_5O_4$: C, 48.72; H, 5.45; Cl, 9.59; N, 18.94;O, 7.31. Found C, 48.59; H, 5.39; N 19.02.

4.2.10. 2-Amino-6-(3-methyl-2-butenylamino)-9-(β-D-ribofuranosyl)-purine (4b)

White solid; 48% yield; R_f = 0.7 (CH_2Cl_2/MeOH, 95:5); mp 210–212 °C; $[\alpha]_D^{20}$ −2.9 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.82 (s, 3H, CH_3), 1.89 (s, 3H, CH_3), 3.72 (d, *J* = 7.0 Hz, 2H, α-CH_2), 3.77 (dd, *J* = 3.1 Hz, *J* = 12.2 Hz, 1H, H5′a), 3.91 (dd, *J* = 2.4 Hz, *J* = 12.2 Hz, 1H, H5′b), 4.17 (ddd, *J* = 2.4 Hz, *J* = 3.1 Hz, *J* = 3.1 Hz, 1H, H4′), 4.34 (dd, *J* = 3.1 Hz, *J* = 5.2 Hz, 1H, H3′), 4.69 (dd, *J* = 5.2 Hz, *J* = 6.0 Hz, 1H, H2′), 5.34 (t, *J* = 7.0 Hz, 1H, β-CH), 5.94 (d, *J* = 6.0 Hz, 1H, H1′), 8.31 (s, 1H, H8); MS (EI): *m/z* 351 (M+1, 100%). Anal. Calcd for $C_{15}H_{22}N_6O_4$: C, 51.42; H, 6.33; N, 23.99; O, 18.27. Found: C, 51.32; H, 6.38, N, 24.01.

4.3. Biological assays

4.3.1. Cell culture

T24 (human bladder carcinoma), were grown in D-MEM medium supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine and 1 mM penicillin/streptomycin (complete growth medium). In some experiments cells were cultured in D-MEM with 0.1% FBS (starvation medium) for 48 h to induce quiescence. This treatment significantly reduced cell growth and ^{3}H-thymidine incorporation (not shown). The cells were cultured in 5% CO_2 at 37 °C. All the reagents for cell culture were from Gibco. Stock solutions of iPA and various compounds were prepared in DMSO and kept at −20 °C. Appropriate dilutions of the compounds were freshly prepared in culture medium just prior the assays. The controls were added with the final concentrations of DMSO (0.01%).

4.3.2. Cell proliferation

For proliferation assays, the cells were seeded at low density (2000/cm^2) in 6-well plates in growth medium. Cells were allowed to attach for 16 h before being exposed to starvation or complete growth medium. After 48 h, the cells were treated with different compounds. On the basis of previous studies, the different molecules were used at a concentration 10 μM.[7] After 72 h, the cells were harvested with trypsin-EDTA and stained with a trypan blue solution. The viable cells were counted using Burker chamber.

In another set of experiments, the different molecules were tested on proliferating or quiescent cells seeded at low density (2000/cm^2) by the MTT reduction assay. This method is based on the reduction of MTT by mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, 16 h after seeding, the cells were exposed either to a starvation medium or to fresh complete medium. 48 h later, the different compounds (10 μM) were added for three additional days.

Before (1 h) the end of treatment the medium was replaced with medium containing 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (Sigma, Oakville, Ontario, Canada). At the end of the incubation, media was removed and formazan crystals generated by the cellular reduction activity were dissolved in DMSO. Absorbance was measured at 575 nm.

4.3.3. Clonogenic assay

5000 T24 cells per well were seeded into 6-well plates and cultured in D-MEM containing 10% FBS supplemented with the compound of interest (10 μM). The medium was replaced every two days and supplemented with fresh compounds. After culture for a week, colonies were fixed in 0.5% crystal violet in methanol and extensively washed. The plates were then photographed.

4.3.4. Migration assay

Cell migration was determined using an in vitro model of wound repair.[18] Briefly, the cells were grown in 24-well plates to confluence. After 24 h starvation in medium with 0.1% FBS, the monolayer was wounded by scratching the surface as uniformly as possible with a pipette tip and then cells were simultaneously incubated with EGF (100 ng/mL) to stimulate cell migration and the different molecules (10 μM) for additional 16 h. This initial wounding and the movement of the cells in the scratched area were photographically monitored using an Axiovert Zeiss 200 microscope with a 10× (0.25 numerical aperture) objective linked to camera. The 16 h-time interval has been chosen because it is shorter than cell doubling time in these conditions. Four different fields from each sample were analysed using the ImageJ software for quantitative estimations of the number of cells that have migrated to the wounded area. The values are expressed as the relative cell migration compared to control condition.

4.3.5. Statistical analysis

All the experiments were performed at least 3 times in triplicates and data are shown as the means ± standard deviation. Statistical significance was determined using the Student's T test. In the figures: $^*p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.093.

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