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N^6-Alkyladenosines: Synthesis and evaluation of in vitro anticancer activity

Roberta Ottria, Silvana Casati, Erika Baldoli, Jeanette A. M. Maier, Pierangela Ciuffreda *

Dipartimento di Scienze Cliniche 'Luigi Sacco', Via G. B. Grassi 74, Università degli Studi di Milano, 20157 Milano, Italy

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ABSTRACT

A series of adenosine analogues differently substituted in N^6-position were synthesized to continue our studies on the relationships between structure and biological activity of iPA. The structures of the compounds were confirmed by standard studies of ^{1}H NMR, MS and elemental analysis. These molecules were then evaluated for their anti-proliferative activity on bladder cancer cells. We found that some of these compounds possess anti-proliferative activity but have no effect on cell invasion and metalloprotease activity.

1. Introduction

Cytokinins, an important group of plant growth regulatory substance, are N^6-substituted adenine derivatives.[1] They occur endogenously as free bases, nucleosides or nucleotides. Cytokinins are often present in a very low concentration and regulate many processes in plants such as bud formation and release, leaf expansion, promotion of seed germination and chloroplast formation.[2] Cytokinins induce callus, a cluster of differentiated plant cells that proliferate indefinitely in a disorganized manner similar to human cancer cells, to re-differentiate into adventitious buds.[3] Since there are some similarities in the biological phenotypes of cancer and callus cells, cytokinins might also affect the growth and differentiation of human cancer cells. The only known cytokinin existing in animal cells is isopentenyladenosine (iPA, **1a**, Fig. 1), a modified nucleoside with a pentaatomic isopentenyl chain that binds the nitrogen at the position 6 of the purinic base. iPA has been detected in the cytosol of many eukaryotic and prokaryotic cells as a free compound or bound to tRNA.[4] It has been demonstrated that iPA exerts a potent anti-proliferative activity on cultured tumour epithelial cells,[5] but it has only a slight effect on tumour growth in rodents.[6] Discouraging results have also been obtained in a pilot clinical trial.[7] The discrepancy between in vitro and in vivo results might be due to the rapid catabolism in vivo, or to the short plasma half-life of iPA, in analogy to other nucleosides. We therefore tried to investigate which structural modifications might yield iPA analogues which maintained their biological activity in vivo. To continue our studies concerning the relation between structure and biological activity of iPA,[8,9] we focused our attention on the isopentenyl chain and synthesised a series of adenosine analogues differently substituted in N^6-position (Fig. 1). In this study, we prepared a group of adenosine derivatives with modification in N^6-chain and we tested them on T24 cells, a cell line established from a human urinary bladder cancer patient, in order to verify whether our

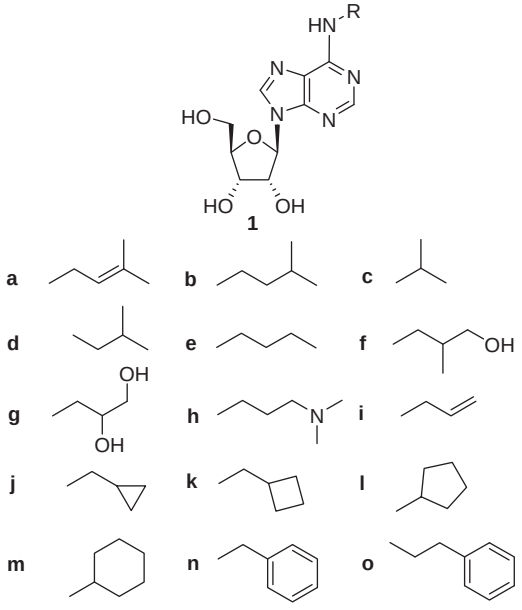

Figure 1. Structures of the modified iPA analogues.

* Corresponding author. Tel.: +39 0250319695; fax: +39 0250319694.
E-mail address: pierangela.ciuffreda@unimi.it (P. Ciuffreda).

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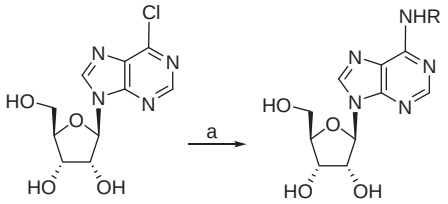

Scheme 1. Synthesis of iPA analogues **1b–o**. Reagents and conditions: (a) appropriate amine, EtOH/Et_3N, 80 °C, 3 h.

modification could affect the anti-proliferative activity of iPA on cell cultures. Bladder cancer is the second most common malignancy of the genitourinary tract. Multifocality and frequent recurrence are the typical characteristics of this tumor.[10] Therefore, novel molecules are needed to treat this neoplasia.

2. Results and discussion

2.1. Chemistry

As part of our continuing efforts to develop iPA analogues endowed with in vivo anti-proliferative activity we turned our attention to compounds characterised by a different substituent in N^6 position (Fig. 1). We synthesised a series of compounds with a saturated linear or ramified chain (**1b–e**), one or more hydroxyl or amino groups on the N^6-substituent (**1f–h**), an unsaturated chain (**1i**), a cyclic substituent (**1j–m**) and an aromatic ring (**1n–o**).

Monosubstituted N^6-alkyladenosines were prepared by condensing 6-chloropurine riboside (6-chloro-9-β-D-ribofuranosyl-9*H*-purine) with the corresponding amines by nucleophilic substitution in absolute ethanol, using triethylamine as acid acceptors (Scheme 1).[11,12] The compounds were isolated and purified by crystallization. The only exception was 6-(cyclobutylmethylamino)-adenosine (**1n**). In this case, cyclobutylmethylbromide was used to alkylate adenosine, and the N^1-alkylated intermediate was rearranged under Dimroth[13,14] conditions to yield **1k**.

The structure of all prepared compounds **1b–o**, were confirmed from MS, 1D and 2D NMR spectroscopic data as well as by elemental (C, H, N) analyses (see Section 4.3) which allowed the correct identification and determined the purity of all compounds.

2.2. Biological assays

Starting from results obtained from dose/response curves performed with iPA,[8] we selected 10 μM as the concentration to utilize to compare iPA (**1a**) and its analogues **1b–o** for their ability to inhibit cell proliferation, clonogenity and invasion. All the compounds were dissolved in DMSO and subsequently diluted in the culture medium before treatment of T24 bladder carcinoma cells, which have been used before to test different iPA analogues.[8,9] To screen all the synthesized compounds for their anti-proliferative capacity, microtiter tetrazolium (MTT) assay was performed. Figure 2a demonstrates a significant anti-proliferative capacity by iPA derivatives **1b**, **1e**, **1i**, **1k** and **1n**. In particular, the activity of compound **1n** is comparable to iPA. These derivatives were tested on another bladder carcinoma cell line, that is, J82, and similar results were obtained (Fig. 2b). Derivatives **1b**, **1e**, **1i**, **1k** and **1n** showed anti-proliferative activity also on colon carcinoma CaCo2 and breast carcinoma MDAMB231 cells (data not shown). The molecules which resulted active by MTT assay were further investigated by T24 cell counting after trypsinization and confirmed to inhibit cell growth (Fig. 2c). Accordingly, clonogenic assays on T24 cells demonstrated that compounds **1b**, **1e**, **1i**, **1k** and **1n** inhibited the formation of clones (Fig. 3). Since tumor cell invasion of basement membranes represents one of the critical steps in the metastatic process, we also investigated whether iPA or its active derivatives modulated cell invasion through basement membrane-like matrigel barriers. In particular, matrigel has been extensively used as an in vitro surrogate for the in vivo process of tumor invasion through basement membranes.[15] Figure 4a shows that iPA and its derivatives **1b**, **1e**, **1i**, **1k** and **1n** did not exert any effect on T24 cell invasion on matrigel. Because increased matrix metalloprotease (MMP) activity has been correlated with the metastatic potential of many cancers,[16] we also analyzed MMP activity by zymography on media collected from T24 cells treated with iPA and its derivatives **1b**, **1e**, **1i**, **1k** and **1n**. A clear band of gelatinolytic activity, sized to 88 kDa corresponding to activated MMP-9, was detected. However, none of the molecules tested altered MMP activity of T24 cells (Fig. 4b). Accordingly, these molecules did not inhibit T24 cell migration in an in vitro model of wound repair (data not shown).

3. Conclusions

Bladder cancer is the fourth most common cancer in men and the five-years relative survival rates are as low as 6% if it is diagnosed at a late stage.[17] Although chemotherapy has improved the treatment of advanced tumors, the associated side effects induced by lack of specificity to tumor cells remain a challenging problem.

Tumor cell lines represent valuable preclinical models to decipher underlying biology and identify potential therapy targets and pharmacologically useful compounds. We have utilized bladder carcinoma T24 cells which have provided important insights into bladder tumour progression events and metastatic dissemination. In these cells we have previously shown that modifications of N^6-position of iPA impact on the cytostatic activity, while modifications of sugar moiety or the purine base generate inactive molecules.[8,9] The anti-proliferative activity is optimal in N^6-substituted adenosine analogues with side chains containing at least three carbon atoms. In addition, the activity is enhanced by the presence in the chain of an unsaturation site and the presence of an heteroatom in the side chain causes a lack in the anti-proliferative activity.

The ability of tumor cells to invade is one of the hallmarks of the metastatic phenotype. We found that iPA **1a** and its derivatives with anti-proliferative activity do not impact on the invasive phenotype of bladder cancer cells. Indeed, no differences between untreated or treated T24 cells were detected using matrigel invasion assay, which shows a strong correlation between the ability of tumor cells to invade in vitro and their invasive behavior in vivo.[15] In addition, because MMPs represent the most prominent family of proteases associated with invasion and metastasis,[16] it is noteworthy that all the compounds tested were unable to modulate MMP activity in T24 cells. We conclude that iPA and derivatives target DNA replication[18] while they do not interfere with the signalling pathways involved in modulating cell invasion.

4. Materials and methods

4.1. General procedures

Melting points were determined with a Stuart Scientific SMP3 melting point apparatus. 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 ^1H. All NMR spectra were recorded at 298 K in CD_3OD (isotopic enrichment 99.95%) solution and the chemical

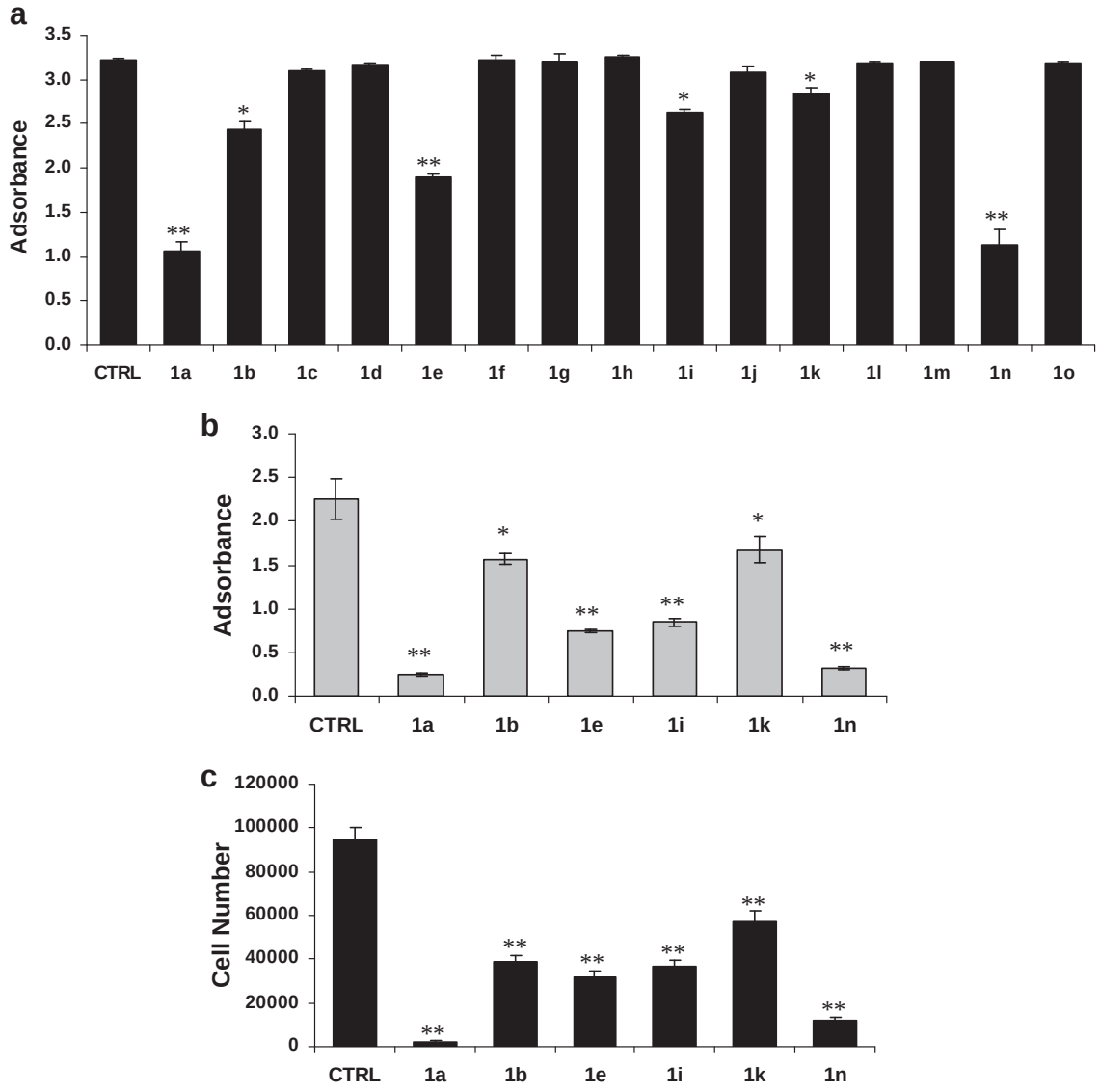

Figure 2. MTT and proliferation assay on bladder carcinoma cells treated with iPA and derivatives. (a) MTT quantification on T24 cells exposed to 10 μM of iPA or synthesized derivatives for 72 h. (b) MTT quantification on J82 cells exposed to 10 μM of iPA or some of its derivatives for 72 h. (c) T24 cells treated with 10 μM iPA or selected derivatives were trypsinized and counted after 72 h of treatment.

shifts were reported on a δ (ppm) scale. The central peak 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 s, broad peak.

Mass spectra were recorded on a Finnigan LCQ deca (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.

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

Purity of all compounds ($\geqslant$99%) was verified by thin-layer chromatography, NMR and Mass Spectrometry measurements.

4.2. Chemicals

Adenosine, 6-chloropurine riboside, all available starting amines, 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.

4.3. Synthesis of N^6-alkyladenosines

To a solution of 6-chloropurine riboside (1.5 mmol) in absolute EtOH (10 mL), Et_3N, (4.5 mmol) and the appropriate amine (4.5 mmol) were added. The mixture was stirred at 80 °C for 3 h, cooled to room temperature and the solvent was removed under vacuum to leave syrupy residue. The addition of dry Et_2O precipitated Et_3NHCl, which was filtered off. The crude residue after evaporation was crystallised from MeOH.

4.3.1. N^6-Isopentyladenosine 1b

Compound **1b** was prepared following the above described procedure starting from 6-chloropurine riboside and 3-methylbutylamine. R_f = 0.48 (CH_2Cl_2/MeOH, 90:10); white solid 78% yield; mp 156–157 °C; $[\alpha]_D^{20}$ −57.8 (*c* 1, MeOH) [lit.[19] mp 154.5–156 °C, $[\alpha]_D^{20}$ −42.0 (*c* 1.03, EtOH)]; ^1H NMR (CD_3OD) δ = 1.0 (d, 6H,

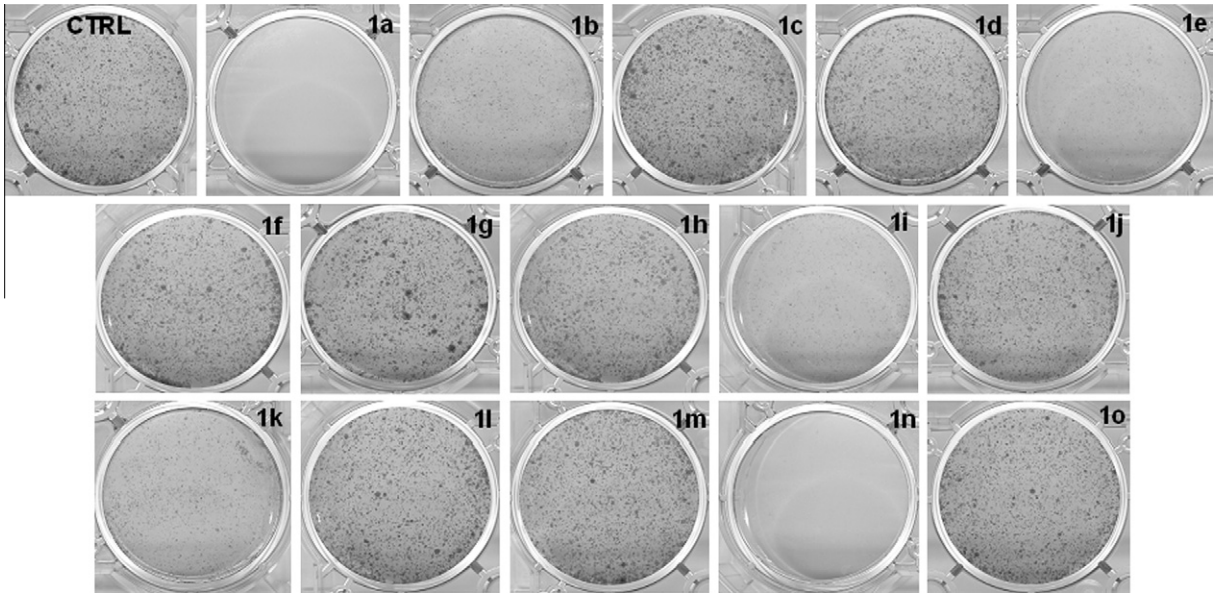

Figure 3. Clonogenic assay on T24 cells treated with iPA and derivatives. T24 cells were seeded at very low density and treated with iPA and derivatives. After a week, the cells were stained and photographed.

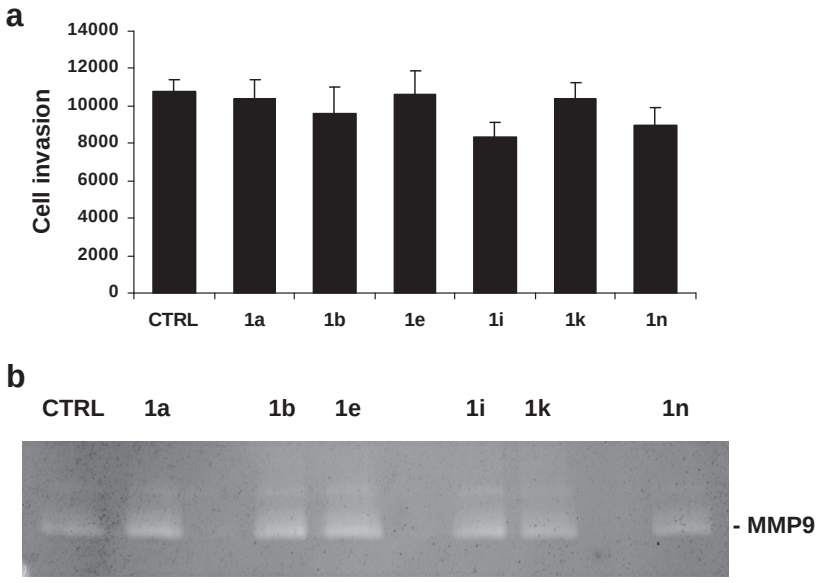

Figure 4. Invasion assay on matrigel and MMP secretion in T24 cells treated with iPA and derivatives. (a) T24 cells were cultured in transwell chambers with Matrigel-coated membranes. Invasion was evaluated as described in Section 4. (b) Zymography was performed on media collected form T24 cells exposed to iPA and derivatives as described in Section 4.

J = 6.6 Hz, CH_3 × 2), 1.61 (dt, 2H, J = 7.0 Hz, J = 7.3 Hz, CH_2CH_2CH), 1.76 (tq, 1H, J = 6.6, J = 7.0, CH_2CH_2CH), 3.63 (br s, 2H, CH_2CH_2CH), 3.76 (dd, 1H, J = 2.4 Hz, J = 12.6 Hz, H5′a), 3.91 (dd, 1H, J = 2.4 Hz, J = 12.6 Hz, H5′b), 4.19 (ddd, 1H, J = 2.4 Hz, J = 2.4 Hz, J = 2.4 Hz, H4′), 4.34 (dd, 1H, J = 2.4 Hz, J = 5.2 Hz, H3′), 4.77 (dd, 1H, J = 5.2 Hz, J = 6.3 Hz, H2′), 5.97 (d, 1H, J = 6.3 Hz, H1′), 8.23 (br s, 1H, H2), 8.25 (s, 1H, H8); ESIMS m/z 336 (M−1, 30%), 673 (2M−1, 100%).

4.3.2. N^6-Isopropyladenosine 1c

Compound **1c** was prepared following the above described procedure starting from 6-chloropurine riboside and 1-methyl-ethylamine. R_f = 0.54 (CH_2Cl_2/MeOH, 90:10); white solid 86% yield; mp 172–174 °C; $[\alpha]_D^{20}$ −21.6 (c 1, MeOH); [lit.[20] mp 157 °C]; ^{1}H NMR (CD_3OD) δ = 1.33 (d, 6H, J = 6.5 Hz, CH_3 × 2), 3.51 (q, 1H, J = 6.5 Hz, NHCH), 3.74 (dd, 1H, J = 2.2 Hz, J = 12.2 Hz, H5′a), 3.90 (dd, 1H, J = 2.2 Hz, J = 12.2 Hz, H5′b), 4.19 (ddd, 1H, J = 2.2 Hz, J = 2.2 Hz, J = 2.5 Hz, H4′), 4.33 (dd, 1H, J = 2.5 Hz, J = 5.9 Hz, H3′), 4.76 (dd, 1H, J = 5.9 Hz, J = 6.3 Hz, H2′), 5.91 (d, 1H, J = 6.3 Hz, H1′), 8.23 (br s, 1H, H2), 8.27 (s, 1H, H8); ESIMS m/z 308 (M−1, 70%), 617 (2M−1, 100%).

4.3.3. N^6-Isobutyladenosine 1d

Compound **1d** was prepared following the above described procedure starting from 6-chloropurine riboside and 2-methyl-propylamine. R_f = 0.60 (CH_2Cl_2/MeOH, 90:10); white solid 84% yield; mp 166–167 °C; $[\alpha]_D^{20}$ −64.0 (c 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.02 (d, 6H, J = 6.5 Hz, CH_3 × 2), 2.01 (tq, 1H, J = 6.5, J = 7.0,

NHCH$_2$C*H*), 3.44 (br s, 2H, NHC*H*$_2$CH), 3.76 (dd, 1H, *J* = 2.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 2.2 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = 2.2 Hz, *J* = 2.2 Hz, *J* = 2.5 Hz, H4′), 4.34 (dd, 1H, *J* = 2.5 Hz, *J* = 5.2 Hz, H3′), 4.76 (dd, 1H, *J* = 5.2 Hz, *J* = 6.5 Hz, H2′), 5.97 (d, 1H, *J* = 6.5 Hz, H1′), 8.23 (br s, 1H, H2), 8.27 (s, 1H, H8); ESIMS *m/z* 322 (M−1, 15%), 645 (2M−1, 100%). Anal. Calcd for $C_{14}H_{21}N_5O_4$: C, 52.00; H, 6.55; N, 21.66; O, 19.79. Found: C, 51.98; H, 6.62; N, 21.49.

4.3.4. N^6-Butyladenosine 1e

Compound **1e** was prepared following the above described procedure starting from 6-chloropurine riboside and *n*-butylamine. R_f = 0.52 (CH_2Cl_2/MeOH, 90:10); white solid 76% yield; mp 176–177 °C; $[\alpha]_D^{20}$ −62.6 (*c* 1, MeOH); [lit.[21] mp 171–173 °C, $[\alpha]_D^{20}$ −36.4 (*c* 1, MeOH)] ^{1}H NMR (CD_3OD) δ = 1.00 (t, 3H, J = 7.7 Hz, C*H*$_3$), 1.49 (qt, 2H, *J* = 6.8 Hz, *J* = 7.7 Hz, NHCH$_2$CH$_2$C*H*$_2$CH$_3$), 1.69 (dt, 2H, *J* = 6.8 Hz, *J* = 7.0 Hz, NHCH$_2$C*H*$_2$CH$_2$CH$_3$), 3.60 (br m, 2H, NHC*H*$_2$CH$_2$CH$_2$CH$_3$), 3.76 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.33 (dd, 1H, *J* = <1.0 Hz, *J* = 5.1 Hz, H3′), 4.76 (dd, 1H, *J* = 5.1 Hz, *J* = 6.4 Hz, H2′), 5.97 (d, 1H, *J* = 6.4 Hz, H1′), 8.23 (br s, 1H, H2), 8.26 (s, 1H, H8); ESIMS *m/z* 322 (M−1, 30%), 645 (2M−1, 100%).

4.3.5. N^6-(3-Hydroxy-2-methylpropyl)-adenosine 1f

Compound **1f** was prepared following the above described procedure starting from 6-chloropurine riboside and (+)-2-amino-1-butanol. R_f = 0.42 (CH_2Cl_2/MeOH, 90:10); white solid 74% yield; mp 192–194 °C; $[\alpha]_D^{20}$ −76.8 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.01 (dd, 3H, *J* = 7.1 Hz, *J* = 7.1 Hz, CH$_2$C*H*$_3$), 1.65 (ddq, 1H, *J* = 7.1 Hz, *J* = 7.1 Hz, *J* = 14.8 Hz, C*H*HCH$_3$), 1.81 (ddq, 1H, *J* = 5.8 Hz, *J* = 7.1 Hz, *J* = 14.8 Hz, CH*H*CH$_3$), 3.70 (d, 2H, *J* = 4.5 Hz, C*H*$_2$OH), 3.76 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.31 (br s, 1H, NHC*H*), 4.34 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.76 (dd, 1H, *J* = 5.2 Hz, *J* = 6.5 Hz, H2′), 5.98 (d, 1H, *J* = 6.5 Hz, H1′), 8.04 (br s, 1H, NH), 8.23 (br s, 1H, H2), 8.27 (s, 1H, H8); ESIMS *m/z* 338 (M−1, 35%), 677 (2M−1, 100%). Anal. Calcd for $C_{14}H_{21}N_5O_5$: C, 49.55; H, 6.24; N, 20.64; O, 23.57. Found: C, 49.48; H, 6.39; N, 20.61.

4.3.6. N^6-2,3-Dihydroxy-propyladenosine 1g

Compound **1g** was prepared following the above described procedure starting from 6-chloropurine riboside and (−)-3-amino-1,2-propanediol. R_f = 0.16 (CH_2Cl_2/MeOH, 90:10); white solid 74% yield; mp 196–198 °C; $[\alpha]_D^{20}$ −48.6 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 3.22 (dd, 1H, *J* = 7.1 Hz, *J* = 13.5 Hz, NHC*H*H), 3.43 (dd, 1H, *J* = 4.5 Hz, *J* = 13.5 Hz, NHCH*H*), 3.59 (d, 2H, *J* = 5.2 Hz, C*H*$_2$OH), 3.71 (ddt, 1H, *J* = 4.5 Hz, *J* = 5.2 Hz, *J* = 7.1 Hz, CH$_2$C*H*OHCH$_2$OH), 3.79 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.87 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.16 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.31 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.73 (dd, 1H, *J* = 5.2 Hz, *J* = 6.5 Hz, H2′), 5.93 (d, 1H, *J* = 6.5 Hz, H1′), 8.08 (br s, 1H, NH), 8.23 (br s, 1H, H2), 8.28 (s, 1H, H8); ESIMS *m/z* 340 (M−1, 15%), 681 (2M−1, 100%). Anal. Calcd for $C_{13}H_{19}N_5O_6$: C, 45.75; H, 5.61; N, 20.52; O, 28.13. Found: : C, 45.62; H, 5.68; N, 20.64.

4.3.7. N^6-3′-(Dimethylamino)propyladenosine 1h

Compound **1h** was prepared following the above described procedure starting from 6-chloropurine riboside and 3-dimethylamino-propylamine. R_f = 0.32 (CH_2Cl_2/MeOH, 90:10); white solid 75% yield; mp 176–178 °C; $[\alpha]_D^{20}$ −33.2 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.89 (m, 2H, NCH$_2$C*H*$_2$CH$_2$N(CH$_3$)$_2$), 2.26 (s, 6H, N(C*H*$_3$)$_2$), 2.46 (m, 2H, NCH$_2$CH$_2$C*H*$_2$N(CH$_3$)$_2$), 3.63 (br s, 2H, NC*H*$_2$CH$_2$CH$_2$N(CH$_3$)$_2$), 3.77 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.18 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.31 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.77 (dd, 1H, *J* = 5.2 Hz, *J* = 6.5 Hz, H2′), 5.93 (d, 1H, *J* = 6.5 Hz, H1′), 8.23 (br s, 1H, H2), 8.26 (s, 1H, H8), 8.57 (br s, 1H, NH); ESIMS *m/z* 351 (M−1, 60%), 703 (2M−1, 100%). Anal. Calcd for $C_{15}H_{24}N_6O_4$: C, 51.13; H, 6.86; N, 23.85; O, 18.16. Found: C, 51.22; H, 6.78; N, 23.74.

4.3.8. N^6-Allyladenosine 1i

Compound **1i** was prepared following the above described procedure starting from 6-chloropurine riboside and allylamine. R_f = 0.40 (CH_2Cl_2/MeOH, 90:10); white solid 75% yield; mp 166–167 °C; $[\alpha]_D^{20}$ −49.6 (*c* 1, MeOH); [lit.[11] mp 166–167 °C; $[\alpha]_D^{25}$ −101 (*c* 0.094, EtOH)]; ^{1}H NMR (CD_3OD) δ 3.77 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.91 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.20 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.25 (br s, 2H, NC*H*$_2$CH), 4.35 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.77 (dd, 1H, *J* = 5.2 Hz, *J* = 6.4 Hz, H2′), 5.18 (d, 1H, *J* = 10.3 Hz, CH=C*H*H), 5.29 (d, 1H, *J* = 16.8 Hz, CH=CH*H*), 5.95–6.10 (m, 1H, CH$_2$C*H*=), 5.99 (d, 1H, *J* = 6.4 Hz, H1′), 8.24 (s, 1H, H2), 8.29 (s, 1H, H8); ESIMS *m/z* 306 (M−1, 40%), 613 (2M−1, 100%).

4.3.9. N^6-Cyclopropylmethyladenosine 1j

Compound **1j** was prepared following the above described procedure starting from 6-chloropurine riboside and cyclopropylmethylamine R_f = 0.42 (CH_2Cl_2/MeOH, 90:10); white solid 80% yield; mp 173–175 °C; $[\alpha]_D^{20}$ −66.4 (*c* 1, MeOH); [lit.[12] mp 176 °C; $[\alpha]_D^{25}$ −64.5 (*c* 0.217, EtOH)]; ^{1}H NMR (CD_3OD) δ = 0.35 (dd, 2H, *J* = 5.8 Hz, *J* = 7.7 Hz, C*H*HCHC*H*H), 0.58 (dd, 2H, *J* = 5.1 Hz, *J* = 5.8 Hz, CH*H*CHCH*H*), 1.17–1.22 (m, 1H, NCH$_2$C*H*), 3.47 (br s, 2H, NC*H*$_2$CH=), 3.77 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.34 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.76 (dd, 1H, *J* = 5.2 Hz, *J* = 6.4 Hz, H2′), 5.97 (d, 1H, *J* = 6.4 Hz, H1′), 8.23 (br s, 1H, H2), 8.28 (s, 1H, H8), 8.57 (br s, 1H, NH); ESIMS *m/z* 320 (M−1, 38%), 641 (2M−1, 100%).

4.3.10. N^6-Cyclobutylmethyladenosine 1k

$BaCO_3$ (2.4 mmol) and cyclobutylmethylbromide (2.25 mmol) were added to a solution of adenosine (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 mixture 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$_2$NH–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 (eluent CH_2Cl_2/MeOH, 98:2). R_f = 0.40 (CH_2Cl_2/MeOH, 95:5); white solid 55% yield; mp 181–183 °C; $[\alpha]_D^{20}$ −42.1 (*c* 1, MeOH); ^{1}H NMR (CD_3OD) δ = 1.81–1.88 (m, 2H, C*H*HCHC*H*H), 1.91–2.00 (m, 2H, CHCH$_2$CH$_2$C*H*$_2$), 2.09–2.17 (m, 2H, CH*H*CHCH*H*), 2.67–2.75 (m, 1H, NCH$_2$C*H*), 3.63 (br s, 2H, NC*H*$_2$CH), 3.76 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.5 Hz, H4′), 4.33 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.76 (dd, 1H, *J* = 5.2 Hz, *J* = 6.4 Hz, H2′), 5.97 (d, 1H, *J* = 6.4 Hz, H1′), 8.23 (br s, 1H, H2), 8.25 (br s, 1H, NH), 8.27 (s, 1H, H8); ESIMS *m/z* 334 (M−1, 25%), 669 (2M−1, 100%). Anal. Calcd for $C_{15}H_{21}N_5O_4$: C, 53.72; H, 6.31; N, 20.88; O, 19.08. Found: C, 53.62; H, 6.40; N, 20.96.

4.3.11. N^6-Cyclopentyladenosine 1l

Compound **1l** was prepared following the above described procedure starting from 6-chloropurine riboside and cyclopentylamine R_f = 0.58 (CH_2Cl_2/MeOH, 98:2); white solid 79% yield; mp 120–122 °C [lit.[22] mp 120–122 °C]; $[\alpha]_D^{20}$ −35.1 (*c* 1, MeOH); ^{1}H

NMR (CD_3OD) δ = 1.60–1.74 (m, 4H, 2 × CH_2), 1.79–1.86 (m, 2H, CH_2), 2.08–2.11 (m, 2H, CH_2), 3.33 (br s, 1H, NC*H*CH_2), 3.76 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.5 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.5 Hz, *J* = 1.5 Hz, H4′), 4.34 (dd, 1H, *J* = <1.0 Hz, *J* = 5.0 Hz, 1H, H3′), 4.75 (dd, 1H, *J* = 5.0 Hz, *J* = 6.6 Hz, H2′), 5.97 (d, 1H, *J* = 6.6 Hz, H1′), 8.23 (br s, 1H, H2), 8.24 (s, 1H, H8); ESIMS *m/z* 334 (M−1, 13%), 669 (2M−1, 100%).

4.3.12. N^6-Cyclohexyladenosine 1m

Compound **1m** was prepared following the above described procedure starting from 6-chloropurine riboside and cyclohexylamine R_f = 0.34 (CH_2Cl_2/MeOH, 95:5); white solid 80% yield; mp 184–186 °C; $[\alpha]_D^{20}$ −60.4 (*c* 1, MeOH); [lit.[12] mp 185 °C; $[\alpha]_D^{25}$ −59 (*c* 0.312, EtOH);]; ^{1}H NMR (CD_3OD) δ = 0.87–0.93 (m, 2H, CH_2), 1.25–1.54 (m, 4H, 2 × CH_2), 1.81–1.86 (m, 2H, CH_2), 2.04–2.08 (m, 2H, CH_2), 3.76 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.90 (dd, 1H, *J* = 1.4 Hz, *J* = 12.2 Hz, H5′b), 4.10–4.14 (br s, 1H, NC*H*CH_2), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.4 Hz, H4′), 4.34 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.76 (dd, 1H, *J* = 5.2 Hz, *J* = 6.5 Hz, H2′), 5.54 (br s, 1H, NH), 5.96 (d, 1H, *J* = 6.5 Hz, H1′), 8.22 (br s, 1H, H2), 8.27 (s, 1H, H8); ESIMS *m/z* 348 (M−1, 22%), 697 (2M−1, 100%).

4.3.13. N^6-Benzyladenosine 1n

Compound **1n** was prepared following the above described procedure starting from 6-chloropurine riboside and benzylamine R_f = 0.63 (CH_2Cl_2/MeOH, 90:10); white solid 76% yield; mp 168–169 °C [lit.[23] mp 167–169 °C]; $[\alpha]_D^{20}$ −68.4 (*c* 0.5, MeOH); ^{1}H NMR (CD_3OD) δ = 3.76 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.81 (br s, 2H, NC*H*$_2$), 3.91 (dd, 1H, *J* = 1.4 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.4 Hz, H4′), 4.34 (dd, 1H, *J* = <1.0 Hz, *J* = 5.2 Hz, 1H, H3′), 4.77 (dd, 1H, *J* = 5.2 Hz, *J* = 6.5 Hz, H2′), 5.51 (br s, 1H, NH), 5.89 (d, 1H, *J* = 6.5 Hz, H1′), 7.25–7.40 (m, 5H, aromatics), 8.26 (br s, 1H, H2), 8.28 (s, 1H, H8); ESIMS *m/z* 356 (M−1, 41%), 713 (2M−1, 100%).

4.3.14. N^6-2-Phenylethyladenosine 1o

Compound **1o** was prepared following the above described procedure starting from 6-chloropurine riboside and 2-phenylethylamine R_f = 0.65 (CH_2Cl_2/MeOH, 90:10); white solid 85% yield; mp 170–171 °C; [lit.[24] mp 166–168 °C]; $[\alpha]_D^{20}$ −28.0 (*c* 0.5, MeOH); ^{1}H NMR (CD_3OD) δ = 3.49–3.53 (m, 2H, NCH_2CH_2), 3.77 (dd, 1H, *J* = 1.2 Hz, *J* = 12.2 Hz, H5′a), 3.86 (br s, 2H, NC*H*$_2$), 3.91 (dd, 1H, *J* = 1.4 Hz, *J* = 12.2 Hz, H5′b), 4.19 (ddd, 1H, *J* = <1.0 Hz, *J* = 1.2 Hz, *J* = 1.4 Hz, H4′), 4.34 (dd, 1H, *J* = <1.0 Hz, *J* = 5.0 Hz, 1H, H3′), 4.77 (dd, 1H, *J* = 5.0 Hz, *J* = 6.6 Hz, H2′), , 5.87 (d, 1H, *J* = 6.6 Hz, H1′), 7.21–7.30 (m, 5H, aromatics), 8.24 (br s, 1H, H2), 8.26 (s, 1H, H8); ESIMS *m/z* 370 (M−1, 39%), 741 (2M−1, 100%).

4.4. Biological assays

4.4.1. Cell culture

T24 and J82 cells were grown in D-MEM or RPMI, respectively, supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine and 1 mM penicillin/streptomycin. 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%).

4.4.2. Cell proliferation

iPA and its derivatives were investigated for their anti-proliferative capacity by MTT reduction assay. On the basis of previous studies, the different molecules were used at a concentration 10 μM.[8] Briefly, 1 h before 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 (MTT) (Sigma, Oakville, Ontario, Canada). At the end of the incubation, media were removed and formazan crystals generated by the cellular reduction activity were dissolved in DMSO. Absorbance was measured at 575 nm and data are expressed as the ratio of absorbance of treated cells versus initial MTT absorbance, corresponding to the level of MTT reduced to formazan crystals by cells in the initial day of treatment.[9]

For proliferation assays, the cells were seeded at low density (2000/cm^2) in 6-well plates and allowed to attach for 16 h before being treated with different compounds.[8,9] 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.

4.4.3. Clonogenic assay

T24 cells (5000 per well) were seeded into 6-well plates and cultured in the presence of iPA or its derivatives (10 μM). The medium was replaced every 2 or 3 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.

4.4.4. Matrigel invasion assay and MMP activity

For the Matrigel invasion assays, 10^5 cells in serum-free culture medium were plated in the top transwell chamber (24-well insert; pore size 8 μm; Greiner bio-one, Germany) with Matrigel-coated membrane (Becton Dickinson, Franklin Lakes, NJ). Medium supplemented with 10% FBS and Epidermal Growth Factor (50 ng/ml) was added to each well of the plate to act as a chemoattractant in the lower chamber.[25] After 24 h, cells on the lower surface of the membrane and cells attached to the surface of the lower chamber were trypsinised and counted. These experiments were conducted in triplicate and performed three times.

For zymography, conditioned media were incubated at 4 °C overnight with gelatin–Sepharose and resolved on an 8% polyacrylamide gels co-polymerized with 1 mg/ml gelatin type B (Sigma Aldrich) under non-reducing conditions without heating. Gels were then washed twice for 30 min in 2.5% Triton X-100 at room temperature and incubated overnight in collagenase buffer (50 mmol/L Tris–HCl, pH 7.5, 10 mmol/L $CaCl_2$, 150 mmol/L NaCl) at 37 °C. Gels were stained in Coomassie Blue R 250 (Bio-Rad, Milano, Italy) in a mixture of methanol–acetic acid–water (4:1:5) for 1 h and destained in the same solution without the dye. The experiment has been performed three times and one representative zymography is shown.

4.4.5. Statistical analysis

All the experiments were performed at least three times in triplicates and data are shown as the mean ± standard deviation. Statistical significance was determined using the Student's *T* test. In the figures: $^{*}p$ <0.05; $^{**}p$ <0.01.

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