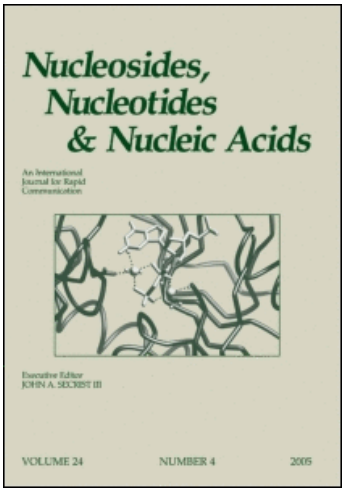

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Ottria, Roberta , Casati, Silvana , Maier, Jeanette A. M. , Mariotti, Massimo and Ciuffreda, Pierangela(2009) 'Novel Isopentenyladenosine Analogues: Synthesis, Characterization, and Evaluation of Antiproliferative Activity on Bladder Carcinoma Cells', Nucleosides, Nucleotides and Nucleic Acids, 28: 8, 736 — 751
To link to this Article: DOI: 10.1080/15257770903155550
URL: http://dx.doi.org/10.1080/15257770903155550

Nucleosides, Nucleotides and Nucleic Acids, 28:736–751, 2009

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770903155550

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NOVEL ISOPENTENYLADENOSINE ANALOGUES: SYNTHESIS, CHARACTERIZATION, AND EVALUATION OF ANTIPROLIFERATIVE ACTIVITY ON BLADDER CARCINOMA CELLS

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□ *Isopentenyladenosine (iPA), a member of the cytokinin family of plant hormones, exerts a marked antiproliferative activity on some leukemic and epithelial cancer cell lines. To characterize the molecular moieties required for the in vitro antitumor activity of the molecule and to obtain cytostatic iPA derivatives potentially useful as chemotherapeutic agents, N9-acyclic analogues have been synthesized using regioselective Mitsunobu reaction and characterized by elemental analyses, ^{1}H and ^{13}C NMR. These compounds were analyzed for their activity on human bladder cancer cell lines. In this study, we report that iPA inhibited the proliferation but not the migration of human bladder cancer cells, while the newly synthesized analogues revealed no significant cytostatic activity apart from the compound with a saturated double bond of the isopentenyl chain. These results indicate that the integrity of the ribose ring is required for the cytostatic activity of iPA.*

Keywords Isopentenyladenosine; Acyclic nucleosides; Antitumor agents; Bladder carcinoma cells

INTRODUCTION

Cytokinins are an important class of plant growth hormones which regulate cell division, differentiation and gene expression.[1] The only known cytokinin existing in animal cells is isopentenyladenosine (iPA, **1**, Figure 1), a modified nucleoside derived from mevalonate. It has been detected in the cytosol of many eukaryotic and prokaryotic cells as a free compound, bound to tRNAs or to a 26 kDa protein.[2] At the moment,

Received 12 May 2009; accepted 9 June 2009.
This work was financially supported by Università degli Studi di Milano (Fondi FIRST).
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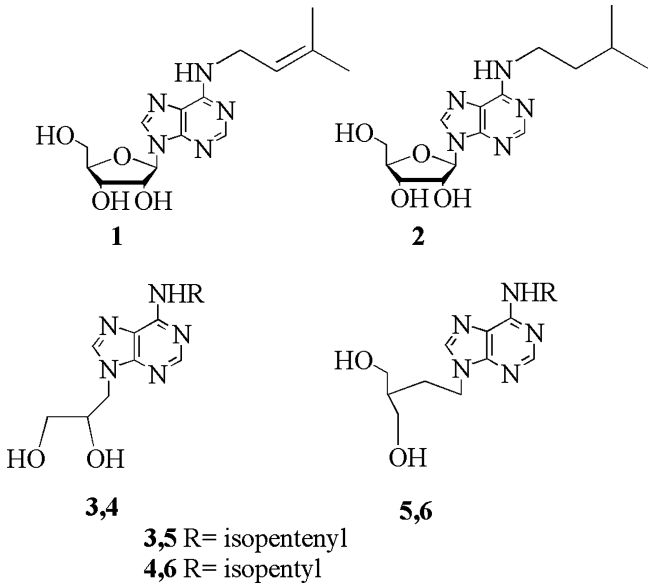


FIGURE 1 The structures of the modified iPA analogous.

however, the biological role of iPA in mammalian cells is rather obscure.[3,4] It has been demonstrated to inhibit protein prenylation and compete for nucleoside transport. In addition, as a component of mammalian serine tRNA, it is conceivable that iPA participates to regulate RNA and protein synthesis.

Spinola et al.[5] have recently shown that iPA exerts potent in vitro antitumor activity on human epithelial cancer cells. This inhibition of cell growth is paralleled by the upregulation of genes involved in arresting cell cycle, as demonstrated by gene expression profile of iPA-treated cancer cells.[6] However, iPA has only slight effect on tumor growth in rodents;[7] this is in agreement with the results obtained in a pilot clinical trial.[8] This lack of effect in vivo might be due to the short plasma half-life of iPA, in analogy to other nucleosides.

With the aim of synthesizing compounds endowed with antiproliferative activity both in vitro and in vivo, we developed structural modifications of iPA. We focused our interest on the modifications of the N^6-isopentenyl moiety as well as on the modifications of the ribosidic moiety.

Initially, the modification of the N^6-isopentenyl moiety with N^6-isopentyl moiety has been considered (isopentyladenosine **2**). Successively, our attention was devoted to the ribosidic portion of iPA, since the β-N-glycosidic bond can be cleaved by enzymatic phosphorolysis in vivo. In this respect, in order to obtain more stable compounds, the β-N-glycosidic bond (N-C-O) was replaced with a stronger N-C-C bond. Therefore, the synthesis of the acyclic nucleosides **3–6**, analogues of the antiviral drug penciclovir,[9]

was considered. The acyclic chain may be indeed considered as resulting from the suppression of a methylene group with respect to a carbohydrate moiety.[10]

Here, we describe the synthesis and biological activity of compound **2–6**. Substituent at position 6 (isopentyl instead of isopentenyl) of adenine and the ribose moiety modification at position 9 were chosen to verify whether these modifications could affect the antiproliferative activity of human bladder cancer cells. Bladder cancer is the second most common malignancy of the genitourinary tract, and the second most common cause of death of all genitourinary tumors. Of all types of cancer, bladder cancer has an unusually high propensity for recurring after therapy, regardless of treatment with surgery, chemotherapy, or immunotherapy. The availability of novel molecules to treat this neoplasia is therefore a crucial topic. This is the reason why we evaluated the activity of iPA and its derivatives on two different human bladder cancer cell lines. We found that iPA was very potent in arresting cell proliferation, while it did not inhibit cell migration, an early pivotal event in cancer invasion. Newly synthesized iPA derivatives, however, were not cytostatic apart from the compound **2** with a saturated double bond of the isopentenyl chain.

RESULTS AND DISCUSSION

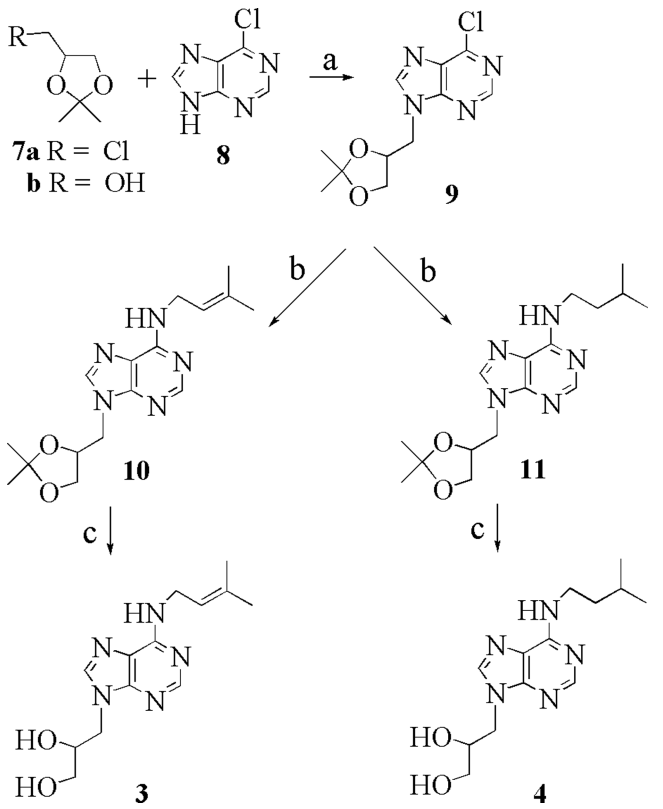
Chemistry

Monosubstituted N^6-isopentyladenosine **2** was prepared in a one-step conversion from 6-chloropurine riboside by nucleophilic substitution with isopentylamine.

For the synthesis of nucleoside analogues in which a modified base was linked to the modified sugar moiety, the N-alkylation of nucleobases has great significance since it is a direct route in the nucleosides synthesis.[11–13] The N-alkylation of purine, or pyrimidine nucleobases, is usually achieved using different sources of carbon electrophiles including: alkyl halides,[14–16] alkyl tosylates,[17] alkyl mesylates,[18] epoxides,[19] Michael acceptors,[20] and allylic esters catalyzed by Pd(0).[21] Finally Mitsunobu conditions were also applied[22–26] due to the advantageous strategy of the direct synthesis of acyclic nucleosides from alcohols.

The first attempt to obtain 6-chloro-9-[(2,2-dimethyl-1,3-dioxan-4-yl)methyl]purine (9) (Scheme 1), key intermediate of compounds **3** and **4**, was the reaction of the chloride **7a** with 6-chloropurine (**8**) and sodium hydride in DMF. Unfortunately, in these conditions, the reaction gave a mixture of undesired by-product 7-isomer and desired product 9-isomer **9** in low yields (8 and 15%, respectively).

Therefore to obtain compound **9**, we tried to introduce the nucleic base by substitution with alcohol **7b** under Mitsunobu conditions. The



SCHEME 1 Reagents and conditions: a) DIAD, THF, Ph_3P, room temperature, 15 hours; b) RNH_2, K_2CO_3, DMF, reflux, 3 hours; c) HCl/MeOH, room temperature, 2 hours.

expected compound **9** was obtained in reasonable yield (64%). It should be noticed that only the N-9 regioisomer formed without another substitution product. The side of N-alkylation was assigned by ^1H and ^{13}C NMR spectra analysis.

Figure 2 shows relevant $^1H/^{13}C$ HMBC correlations for the identification of compounds **9** as the N-9 regioisomers, in particular the diagnostic HMBC correlations between CH_2 or CH protons of the acyclic group with H8, H5, and H2. In the ^1H NMR spectrum of **9** the signal of proton H2 was unequivocally assigned by detecting the three-bond interaction with C4 and C6 (HMBC), whereas the signal of H8 was coupled by three-bond scalar interaction with C4 and C5.

The stereochemistry of **9** was also elucidated by NOE correlations observed between H2 and CH and CH_2 of the acyclic moiety (Figure 3).

Monosubstituted N^6-isopentenyl derivative **10** and N^6-isopentyl derivative **11** were prepared in a one step conversion from **9** by nucleophilic substitution with isopentenylamine and isopentylamine in almost quantitative yields. These compounds were successively deprotected with

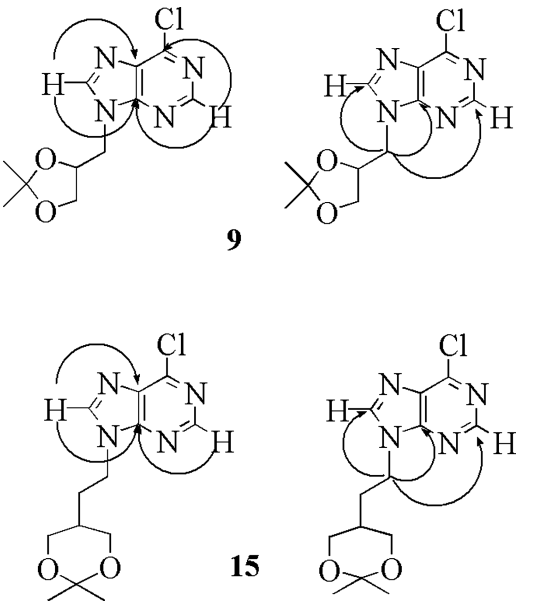


FIGURE 2 Relevant $^{1}H/^{13}C$ HMBC correlations for identification of compounds **9** and **15** as the N-9 regioisomers.

hydrochloric acid in methanol at room temperature to obtain the target compounds **3** and **4** in 67 and 79% yields, respectively. The overall chemical yield from **8** was 43% for compound **3** and 50% for **4**.

The synthesis of the 1,3-protected triol **13**, essential for the achievement of compounds **5** and **6**, was performed modifying the procedures reported in literature (Scheme 2).[9]

The commercially available starting material triethyl-1,1,2-ethanetricarboxylate (**12**) was converted in 2-(hydroxymethyl)butane-1,4-diol (**13**) through a reduction reaction with an excess of sodium borohydride in *tert*-butyl alcohol in 90% yield. The crude product contained a polar contaminant so it was convenient to purify it by column

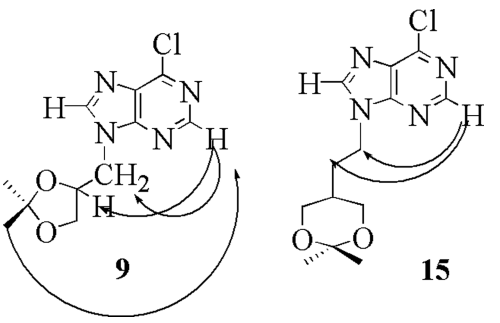
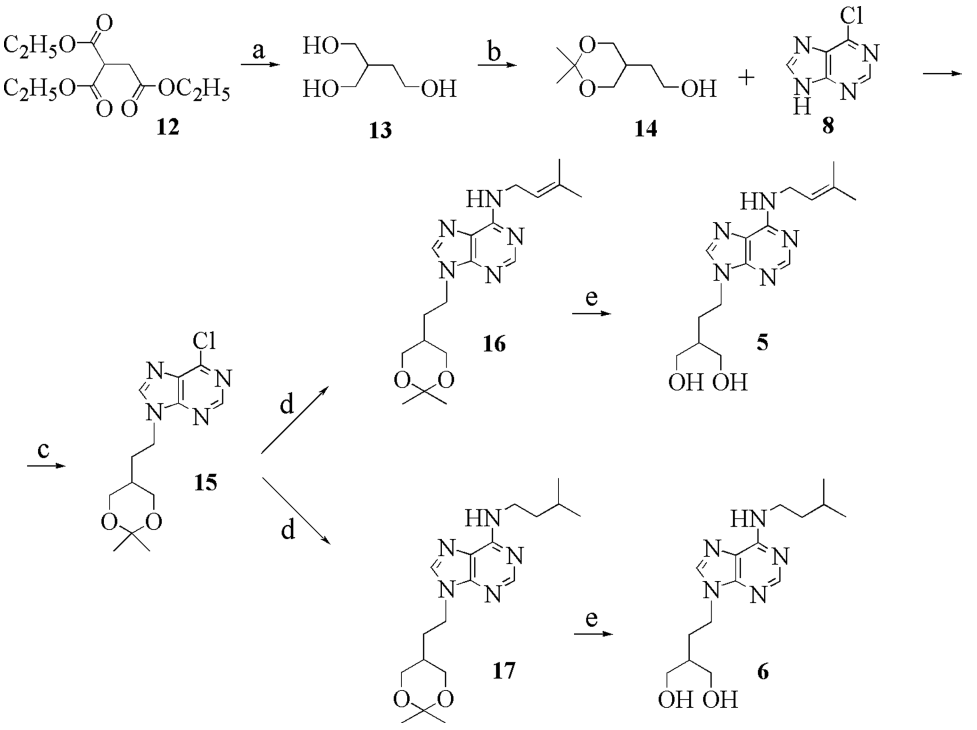


FIGURE 3 NOE correlations in compounds **9** and **15**.



SCHEME 2 Reagents and conditions: a) $NaBH_4$, *t*BuOH, MeOH reflux for 60 minutes and room temperature overnight; b) 2,2-dimethoxypropane, H_2SO_4, acetone, room temperature, 19 hours; c) DIAD, THF, Ph_3P, room temperature, 15 hours; d) RNH_2, K_2CO_3, DMF, reflux, 3 hours; e) HCl/MeOH, room temperature, 2 hours.

chromatography. Lithium aluminum hydride (LAH) attempt to reduce the triester, resulted in a mixture of two unidentified components. The selective 1,3-protection of the triol **13** was completed by the introduction of the isopropylidene group through the reaction of **13** with 2,2-dimethoxypropane catalyzed by sulfuric acid. The reaction took 19 hours to produce 5-(2-hydroxyethyl)-2,2-dimethyl-1,3-dioxane (**14**) in 57% yield. A small amount of 7-membered ring by-product, very close in TLC to the desired 6-membered ring product **14**, also formed. It was therefore necessary a particularly careful column chromatography to purify compound **14**.

The alcohol **14** was treated with 6-chloropurine (**8**), triphenylphosphine and DIAD under Mitsunobu conditions (Scheme 2). The expected compound **15** was obtained in reasonable yield (72%). It should be noticed that only the N-9 regioisomer formed. The side of N-alkylation was assigned by detecting the HMBC and NOESY interactions as analogously reported for the compound **9** (Figure 2 and Figure 3).

Monosubstituted N^6-isopentenyl derivative **16** and N^6-isopentyl derivative **17** were prepared in a one step conversion from **15** by nucleophilic

substitution with isopentenylamine and isopentylamine in nearly quantitative yields. These compounds were then deprotected with hydrochloric acid in methanol at room temperature, to obtain the target compounds **5** (67% yield) and **6** (70% yield). The overall chemical yield from **8** was 48% for compound **5** and 50% for **6**.

Effects of IPA Analogues on Human Bladder Cancer Cell Lines

Two different cell lines derived from human transitional cell bladder carcinomas were selected for these studies: non tumorigenic T24 and tumorigenic J82. We first evaluated the effects of iPA on the proliferation of these cells. After 72 hours in the presence of different concentrations of iPA, the cells were trypsinized and counted. We found that iPA inhibits cell growth in a dose dependent fashion (Figure 4a).

To test whether this inhibition was reversible or not, after 12 hours exposure to iPA (10 μM), we removed the compound, washed the monolayer and incubated the cells in their culture medium for additional 60 hours. Figure 4b shows that iPA inhibitory effect was irreversible both in T24 and in J82 cells. We then compared iPA and its analogues **2–6** for their capability to inhibit cell growth. Because the concentration of iPA which exerted the maximal effect is 10 μM, the cells were exposed to 10 μM of each molecule for 72 hours. Apart from compound **2**, which showed a partial inhibitory activity, all the analogues had no effect on cell growth both in J82 and in T24 cells (Figure 4c). Consistently, whereas iPA completely inhibited type clonogenity after 10 days of treatment, compound 2 reduced the number of colonies and the other molecules had no effect on the clonogenicity of T24 and J82 cells (Figure 5).

We also evaluated the effects of iPA on cell migration, which is fundamental for invasion and metastasis. T24 cells were treated for 8 hours with iPA and then exposed to EGF (10 ng/mL) for 14 additional hours. iPA and its derivatives exerted no effect on basal or EGF-stimulated migration in T24 cells (Figure 6). Similar results were obtained in J82 cells (not shown).

CONCLUSION

Carcinoma of the bladder is the fifth most frequent human cancer, primarily affecting people over the age of 50 years, and accounts for approximately 3% of all cancer-related deaths.[27] It might aggressively invade the bladder wall and originate metastases. These tumors frequently recur after excision; consequently, it is relevant to search for novel molecules that could prevent their recurrence and/or inhibit their progression. Our results demonstrate that iPA is a potent irreversible inhibitor of bladder cancer cell growth, while it does not exert any activity on cell migration, which is necessary to invade the surrounding tissues and, eventually, to

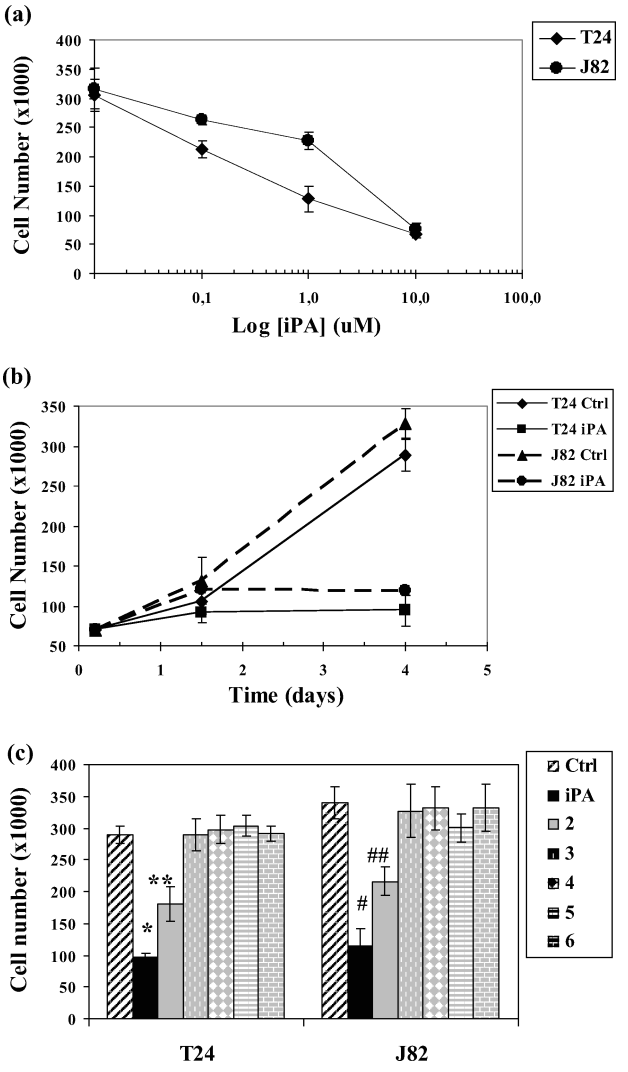


FIGURE 4 Effects of iPA and its derivatives on bladder cancer cell growth. a) T24 and J82 cells were cultured in the presence or in the absence of different concentrations of iPA. Seventy-two hours later, the cells were trypsinized and counted as described in the methods. Results are expressed as the mean ± standard deviation. b) Twenty-four hours after seeding, the cells were exposed to iPA (10 μM) for 12 hours. Then iPA was removed and the cells incubated for an additional 60 hours in their culture medium before being counted as described above. c) T24 and J82 cells were cultured in the presence or in the absence of iPA and its derivatives (10 μM). The experiment was performed as described in A. $^{*}p = 0.0036$, $^{**}p = 0.015$, $\#p = 0.0044$, $\#\#p = 0.0041$.

generate metastases. Interestingly, iPA was reproducibly unable to modulate cell motility of several other cancer cell lines. On these bases, we propose that iPA interferes with the complex regulation of cell proliferation without affecting the signalling pathways necessary for cell migration.

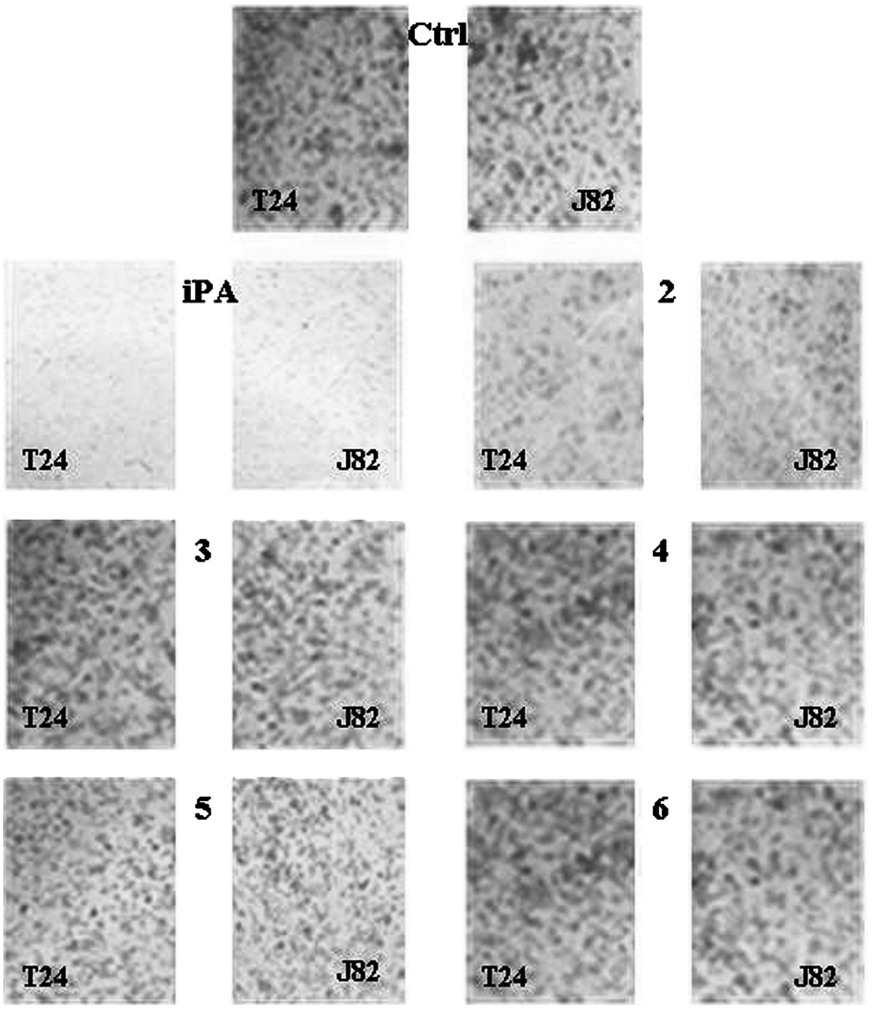


FIGURE 5 Effects of iPA and its derivatives on the clonogenic activity. T24 and J82 cells were seeded at very low density, treated with iPA and derivatives for 72 hours and maintained thereafter in culture medium alone until the end of the experiment. The cells were stained and photographed (magnification 40 ×).

In spite of its inhibitory effects in vitro, iPA is not active in vivo,[7] probably because it is rapidly inactivated. For this reason, iPA derivatives were synthesized and tested for their activity on bladder cancer cell lines. Various iPA analogues, modified at position 9, were synthesized by direct coupling using Mitsunobu reaction that provided the expected compounds in an efficient way, leading only to the N-9 adenine analogue. These derivatives were evaluated for their *in vitro* effects, but no significant effect was detected both on cell migration and on cell growth. In particular, when the lateral chain was modified, a less active compound was obtained (compound

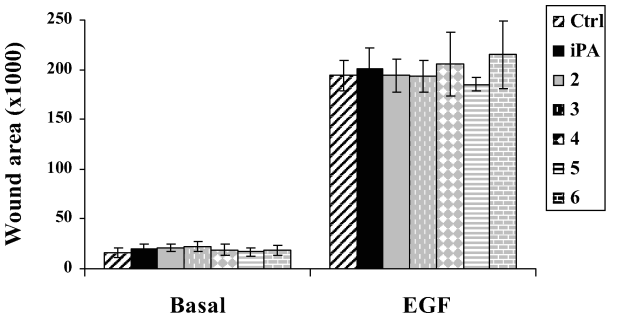


FIGURE 6 Effects of iPA and its derivatives on bladder cancer cell migration. Wound assay was performed on confluent T24 cells. The cells were treated with EGF (100 ng/ml) and the different molecules for 16 hours. The wound area was calculated by a specific software and expressed using an arbitrary value scale.

2), whereas the substitution of the ribose moiety gave completely inactive compounds (**3–6**).

We conclude that the antiproliferative action of iPA seems to depend on two factors, namely, the ribose moiety and the substitution of amine group in position 6. Further studies are necessary to identify the chemical modifications which might preserve the biological activity also in vivo.

EXPERIMENTAL

General

Melting points were determined with a Stuart Scientific SMP3 melting point apparatus and left uncorrected. Optical rotations were measured on a 241 polarimeter (sodium D line at 25°C; Perkin Elmer, Waltham, MA, USA). NMR spectra were done on a Bruker (Rheinstetten, Germany) AVANCE 500 spectrometer equipped with a 5mm broadband reverse probe with field *z*-gradient operating at 500.13 and 125.76 MHz for ^{1}H and ^{13}C, respectively. 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 for ^{1}H and 77.7 ppm for ^{13}C) and of CD_3OD signals (3.31 ppm for ^{1}H and 49.1 ppm for ^{13}C) were used as internal reference standard. (NMR descriptions; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, and bs, broad singlet). Mass spectra were recorded on a Finnigan LCQdeca (TermoQuest) 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 on all intermediates and are within ± 0.4% of theoretical values.

(*S*)-(+)-2,2-Dimethyl-1,3-dioxolane-4-methanol (**7b**), dimethylallylbromide, 1-amino-3-methylbutane and 6-chloropurine riboside, 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.

3,3-dimethylallylamine was synthesized starting from 3,3-dimethyl allylbromide according to literature method.[28]

Syntheses

General Procedure for Mitsunobu Reaction

Diisopropyl azodicarboxylate (DIAD, 1.2 mmol) was added dropwise over 3 hours to a suspension of 6-chloropurine (**8**) (1 mmol), triphenylphosphine (1.2 mmol) and suitably protected alcohol (1.2 mmol) in dry acetonitrile (20 mL), under argon atmosphere at 0°C. The resulting solution was stirred at room temperature overnight to yield a light orange solution. Evaporation of the solvent gave a residue that was submitted to column chromatography on silica gel (CH_2Cl_2/MeOH) to afford the corresponding acyclonucleoside.

General Procedure for 6-Chlorine Substitution and Acyclic Chain Deprotection

K_2CO_3 (4.5 mmol) and the appropriate amine (4.5 mmol) were added to a solution of 6-chloropurine derivative (1.5 mmol) in DMF (20 mL). The mixture was refluxed for 3 hours, cooled to room temperature, filtered on celite, and the solvent was removed under vacuum. The crude residue was stirred in HCl (1.0 M in MeOH, 2.2 mL) at room temperature for 2 hours. The solution was neutralized by addition of DOWEX 50WX8–200. The basic resin was filtered off, the solvent removed and the residue purified by column chromatography on silica gel.

6-(3-Methylbutyl)-9-(β-D-ribofuranosyl)purine (2)

Compound **2** was prepared following the above described procedure starting from 6-chloropurine riboside and 1-amino-3-methylbutane. R_f = 0.48 (CH_2Cl_2/MeOH, 90:10); white solid 68% yield; m.p. 152–154°C; $[\alpha]^{20}_D$ -58.7 (c 1, MeOH) [lit.[29] m.p. 154.5–156°C, $[\alpha]^{20}_D$ -42 (c 1.03, EtOH)]; ^{1}H NMR (CD_3OD) δ = 1.00 (d, J = 6.6 Hz, 6H, 2 × CH_3), 1.60 (dt, J = 6.6 Hz, J = 6.6 Hz, 2H, β-CH_2), 1.76 (dq, J = 6.6 Hz, J = 6.6 Hz, 1H, γ-CH), 3.63 (bs, 2H, α-CH_2), 3.76 (dd, J = 2.5 Hz, J = 12.6 Hz, 1H, H5′a), 3.91 (dd, J = 2.5 Hz, J = 12.6 Hz, 1H, H5′b), 4.19 (ddd, J = 2.5 Hz, J = 2.5 Hz, J = 2.5 Hz, 1H, H4′), 4.34 (dd, J = 2.5 Hz, J = 5.2 Hz, 1H, H3′), 4.76 (dd, J = 5.2 Hz, J = 6.3 Hz, 1H, H2′), 5.97 (d, J = 6.3 Hz, 1H, H1′), 8.23 (s, 1H,

H2), 8.25 (s, 1H, H8); ^{13}C NMR δ = 19.98 (2 × CH_3), 24.06 (γ-CH), 36.50 (α-CH_2), 37.00 (β-CH_2), 60.62 (C5′), 69.81 (C4′), 72.55 (C2′), 85.32 (C3′), 88.41(C1′), 120.33 (C5), 138.47 (C8), 147.66 (C4), 150.66 (C2), 153.45 (C6); ESIMS *m/z* 336 (M-1, 30%), 673 (2M-1, 100%).

6-Chloro-9-[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]purine (9)

Compound **9** was prepared following the above described procedure starting from 6-chloropurine (**8**) and (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol (**7b**). R_f = 0.39 (CH_2Cl_2/MeOH, 98:2); amorphous foam; 64% yield; m.p. 98–100°C; ^{1}H NMR ($CDCl_3$) δ = 1.33 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 3.72 (dd, J = 5.5 Hz, J = 9.0 Hz, 1H, CH_2-O), 4.15 (dd, J = 6.2 Hz, J = 9.0 Hz, 1H, CH_2-O), 4.37 (dd, J = 6.9 Hz, J = 15.2 Hz, 1H, N-CH_2), 4.46 (dd, J = 3.5 Hz, J = 15.2 Hz, 1H, N-CH_2), 4.51 (dddd, J = 3.5 Hz, J = 5.5 Hz, J = 6.2 Hz, J = 6.9 Hz, 1H, CH-O), 8.27 (s, 1H, H8), 8.74 (s, 1H, H2); ^{13}C NMR δ = 24.92 (CH_3), 26.63 (CH_3), 46.19 (N-CH_2), 66.30 (CH_2-O), 73.55 (CH-O), 110.38 ($C(CH_3)_2$), 131.28 (C5), 146.32 (C8), 151.12 (C4), 151.28 (C6), 151.96 (C2); Anal. calcd for $C_{11}H_{13}ClN_4O_2$: C, 49.17; H, 4.88; Cl, 13.19; N, 20.85; O, 11.9. Found: C, 49.29; H, 4.92; Cl, 13.22; N, 20.65.

6-(3-Methyl-2-butenylamino)-9-(propane-2,3-diol-1-yl)purine (3)

Compound **3** was prepared following the above described procedure starting from 6-chloro-9-[(2,2-dimethyl-1,3-dioxan-4-yl)methyl]purine (**9**) and 3,3-dimethylallylamine to give 6-(3-methyl-but-2-enylamino)-9-[(2,2-dimethyl-1,3-dioxan-4-yl)-methyl]purine (**10**) that was deprotected to give the desired compound **3**. R_f = 0.21 (CH_2Cl_2/MeOH, 90:10); white solid; 67% yield; m.p. 154–156°C; $[\alpha]^{20}_D$ -23.2 (c 1, MeOH); ^{1}H NMR ($CDCl_3$) δ = 1.77 (s, 3H, CH_3), 1.79 (s, 3H, CH_3), 3.49 (dd, J = 5.5 Hz, J = 11.8 Hz, 1H, CH_2-OH), 3.57 (dd, J = 4.2 Hz, J = 11.8 Hz, 1H, CH_2-OH), 4.09 (dddd, J = 4.2 Hz, J = 4.2 Hz, J = 5.5 Hz, J = 5.5 Hz, 1H, CH-OH), 4.23 (bs, 2H, α-CH_2), 4.34 (dd, J = 4.2 Hz, J = 14.5 Hz, 1H, N-CH_2), 4.39 (dd, J = 5.5 Hz, J = 14.5 Hz, 1H, N-CH_2), 5.40 (t, J = 7.0 Hz, 1H, β-CH), 5.81 (bs, 1H, NH), 7.74 (s, 1H, H8), 8.38 (s, 1H, H2); ^{13}C NMR δ = 18.00 (CH_3), 25.66 (CH_3), 38.75 (α-CH_2), 46.78 (N-CH_2), 62.77 (CH_2-OH), 70.62 (CH-OH), 119.96 (β-CH), 136.85 (γ-C), 137.00 (C5), 140.63 (C8), 151.00 (C4), 152.98 (C2), 154.78 (C6); ESIMS *m/e* 276 (M-1, 100%), 553 (2M-1, 31%); Anal. calcd for $C_{16}H_{23}N_5O_2$: C, 60.55; H, 7.30; N, 22.07; O, 10.08. Found: C, 60.43; H, 7.38; N, 22.17.

6-(3-Methyl-butylamino)-9-(propane-2,3-diol-1-yl)purine (4)

Compound **4** was prepared following the above described procedure starting from 6-chloro-9-[(2,2-dimethyl-1,3-dioxan-4-yl)methyl]purine (**9**) and 1-amino-3-methylbutane to give 6-(3-methyl-butylamino)-9-[(2,2-dimethyl-1,3-dioxan-4-yl)-methyl]purine (**11**) that was deprotected to the desired compound **4**. R_f = 0.23 (CH_2Cl_2/MeOH, 90:10); white solid; 69%

yield; m.p. 98–100°C; $[\alpha]^{20}_D$ -20.0 (c 1, MeOH); ^{1}H NMR ($CDCl_3$) $\delta = 0.98$ (d, $J = 6.6$ Hz, 6H, 2 × CH_3), 1.60 (dt, $J = 6.6$ Hz, $J = 6.6$ Hz, 2H, β-CH_2), 1.76 (dq, $J = 6.6$ Hz, $J = 6.6$ Hz, 1H, γ-CH), 3.50 (dd, $J = 6.2$ Hz, $J = 11.8$ Hz, 1H, CH_2-OH), 3.58 (dd, $J = 4.2$ Hz, $J = 11.8$ Hz, 1H, CH_2-OH), 3.66 (bs, 2H, α-CH_2), 4.09 (dddd, $J = 4.2$ Hz, $J = 4.2$ Hz, $J = 5.5$ Hz, $J = 6.2$ Hz, 1H, CH-OH), 4.32–4.40 (AB part of ABX system, 2H, N-CH_2), 6.05 (bs, 1H, NH), 7.75 (s, 1H, H8), 8.35 (s, 1H, H2); ^{13}C NMR $\delta = 22.50$ (2 × CH_3), 25.78 (γ-CH), 38.43 (α-CH_2), 39.00 (β-CH_2), 46.79 (N-CH_2), 62.76 (CH_2-OH), 70.57 (CH-OH), 139.23 (C5), 140.60 (C8), 151.98 (C4), 153.01 (C2), 154.88 (C6); ESIMS *m/z* 278 (M-1, 100%), 557 (2M-1, 52%); Anal. calcd for $C_{16}H_{25}N_5O_2$: C, 60,17; H, 7.89; N, 21.93; O, 10.02. Found: C, 60,09; H, 7.76; N, 22.06.

2-(Hydroxymethyl)butane-1,4-diol (13)[9]

Triethyl-1,1,2-ethanetricarboxylate (**12**) (4.65 mL, 20.3 mmol) and $NaBH_4$ (2.0 g, 53 mmol) were dissolved in *tert*-butylalcohol (40 mL). The mixture was heated to reflux and then MeOH (2.5 mL) was added in portions. The mixture was refluxed for 1 additional hour, then cooled to room temperature and stirred overnight. HCl (5M, 3 mL) was added carefully until the solution was neutral. A large amount of white solid formed. The mixture was filtered and the residue was washed with EtOH (30 mL), after removal of the solvent the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 95:5) to give a colorless oil **13** (2.17 g, 91%); $R_f = 0.35$ (CH_2Cl_2/MeOH, 95:5); ^{1}H NMR (DMSO) $\delta =$ 1.39 (q, $J = 6.6$ Hz, 2H, CH-*CH_2*), 1.54–1.58 (m, 1H, *CH*-CH_2), 3.33–3.39 (m, 2H, ax-CH), 4.42–4.45 (m, 2H, eq-CH).

5-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxane (14)[9]

Sulfuric acid 96% (0.2 mL) was added to a solution of 2-(hydroxymethyl)butane-1,4-diol (**13**) (1 g, 8.4 mmol) and 2,2-dimethoxypropane (2 mL, 16.3 mmol) in acetone (5 mL) and the mixture was stirred overnight at room temperature. $Ca(OH)_2$ was added until the solution was neutral, the mixture was filtered, and the solvent removed under vacuum. The residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 95:5) to give **14** as a colorless oil (0.450 g, 33%); $R_f = 0.35$ (CH_2Cl_2/MeOH, 95:5); ^{1}H NMR ($CDCl_3$) $\delta = 1.44$ (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 1.58 (dt, $J = 6.2$ Hz, $J = 6.2$ Hz, 2H, CH-*CH_2*), 1.95–2.00 (m, 2H, *CH*-CH_2), 3.66 (dd, $J = 8.2$ Hz, $J = 12.2$ Hz, 2H, ax-CH), 3.74 (t, $J = 6.3$ Hz, 2H, CH_2OH), 3.96 (dd, $J = 4.6$, $J = 12.2$ Hz, 2H, eq-CH).

6-Chloro-9-[(2,2-dimethyl-1,3-dioxan-5-yl)ethyl]purine (15)

Compound **15** was prepared following the above described procedure starting from 6-chloropurine (**8**) and 5-(2-hydroxyethyl)-2,2-dimethyl-1,3-dioxane (**14**). $R_f = 0.4$ (CH_2Cl_2/MeOH, 95:5); white solid; 72% yield; m.p.

146–148°C; [lit.[19] m.p. 148–150°C]; ^{1}H NMR ($CDCl_3$) δ = 1.45 (s, 6H, 2 × CH_3), 1.70–1.76 (m, 1H, *CH*-CH_2), 2.06 (dt, J = 6.9 Hz, J = 6.9 Hz, 2H, CH-*CH_2*), 3.70 (dd, J = 6.9 Hz, J = 11.8 Hz, 2H, CH_2-O), 4.01 (dd, J = 4.2 Hz, J = 11.8 Hz, 2H, CH_2-O), 4.38 (t, J = 6.9 Hz, 2H, N-CH_2), 8.16 (s, 1H, H2), 8.78 (s, 1H, H8).

6-(3-Methyl-2-butenylamino)-9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]purine (5)

Compound **5** was prepared following the above described procedure starting from 6-chloro-9-[(2,2-dimethyl-1,3-dioxan-5-yl)ethyl]purine (**15**) and 3,3-dimethylallylamine to give 6-(3-methyl-but-2-enylamino)-9-[(2,2-dimethyl-1,3-dioxan-5-yl)ethyl]purine (**16**) that was deprotected to the desired compound **5**. R_f = 0.18 (CH_2Cl_2/MeOH, 90:10); white solid; 67% yield; m.p. 104–106°C; $[\alpha]^{20}_D$ -00.0 (c 1, MeOH); ^{1}H NMR ($CDCl_3$) δ = 1.75 (s, 3H, CH_3), 1.76 (m, overlapped, 1H, *CH*-CH_2-OH), 1.77 (s, 3H, CH_3), 2.01 (dt, J = 6.9 Hz, J = 6.9 Hz, 2H, CH-*CH_2*), 3.74–3.80 (m, 4H, 2 × CH_2-OH), 4.22 (bs, 2H, α-CH_2), 4.34 (t, J = 6.9 Hz, 2H, N-CH_2), 5.38 (t, J = 7.0 Hz, 1H, β-CH), 5.93 (bs, 1H, NH), 7.76 (s, 1H, H8), 8.37 (s, 1H, H2); ^{13}C NMR δ = 18.02 (CH_3), 25.69 (CH_3), 29.33 CH-*CH_2*), 38.70 (α-CH_2), 39.87 (*CH*-CH_2-OH), 42.03 (N-CH_2), 64.35 (CH_2-OH), 120.05 (β-CH), 136.95 (γ-C), 137.00 (C5), 139.60 (C8), 152.13 (C4), 153.01 (C2), 154.70 (C6); ESIMS *m/z* 304 (M-1, 100%), 609 (2M-1, 50%); Anal. calcd for $C_{15}H_{23}N_5O_2$: C, 59.00; H, 7.59; N, 22.93; O, 10.48. Found: C, 58.88; H, 7.42; N, 22.98.

6-(3-Methyl-butylamino)-9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]purine (6)

Compound **6** was prepared following the above described procedure starting from 6-chloro-9-[(2,2-dimethyl-1,3-dioxan-5-yl)ethyl]purine (**15**) and 1-amino-3-methylbutane to give 6-(3-methyl-butylamino)-9-[(2,2-dimethyl-1,3-dioxan-5-yl)ethyl] purine (**17**) that was deprotected to the desired compound **6**. R_f = 0.22 (CH_2Cl_2/MeOH, 90:10); white solid; 70% yield; m.p. 96–98°C; α_D 0.00 (c = 1 in MeOH); ^{1}H NMR ($CDCl_3$) δ = 0.99 (d, J = 6.6 Hz, 6H, 2 × CH_3), 1.60 (dt, J = 6.6 Hz, J = 6.6 Hz, 2H, β-CH_2), 1.77 (dq, J = 6.6 Hz, J = 6.6 Hz, 1H, γ-CH), 2.04 (dt, J = 6.9 Hz, J = 6.9 Hz, 2H, CH-*CH_2*), 3.68 (bs, 2H, α-CH_2), 3.76–3.83 (m, 4H, 2 × CH_2-OH), 4.36 (t, J = 6.9 Hz, 2H, N-CH_2), 5.86 (bs, 1H, NH), 7.76 (s, 1H, H8), 8.38 (s, 1H, H2); ^{13}C NMR δ = 22.52 (2 × CH_3), 25.77 (γ-CH), 29.38 (CH-*CH_2*), 38.53 (β-CH_2), 38.98 (α-CH_2), 39.74 (*CH*-CH_2-OH), 41.96 (N-CH_2), 64.68 (CH_2-OH), 119.80 (C5), 139.51 (C8), 148.67 (C4), 153.08 (C2), 155.04 (C6); ESIMS *m/z* 306 (M-1, 100%), 613 (2M-1, 72%); Anal. calcd for $C_{15}H_{25}N_5O_2$: C, 58.61; H, 8.20; N, 22.78; O, 10.41. Found: C, 58.46; H, 8.12; N, 22.88.

Cell Culture, Proliferation, and Migration

T24 and J82 cells were cultured in DMEM containing 10% calf serum. All the reagents for cell culture were from Gibco Invitrogen Co. (Scotland, UK). Stock solutions of iPA (**1**) and compounds **2–6** were prepared in DMSO and kept at −20°C. Appropriate dilutions of the compounds were freshly prepared just prior the assays. The controls were additioned with the final concentrations of DMSO.

Cell migration was determined using an *in vitro* model of wound repair as previously described.[30] Briefly, the cells were grown in 24-well plates to confluence. After an overnight of starvation in DMEM containing 0.1% serum, the monolayer was wounded, EGF (100 ng/ml) and the different molecules (10 μM) were added for an additional 16 hours. The wound area was calculated by the ImageJ software and expressed using an arbitrary value scale. For proliferation assays, the cells were seeded at low density in growth medium.[30] Sixteen hours after plating, iPA and its derivatives were added. Seventy-two hours later, the cells were trypsinized, stained with a trypan blue solution (0,4%) and the viable cells were counted using a Burker chamber. In some experiments iPA was added for 12 hours, washed, and the cells were maintained in culture medium for 60 hours. For clonogenic assays, T24 and J82 cells were plated at a density of 500 cells/well in 6 well-plates and treated with iPA and its analogues (10 μM) for 10 days. Plates were then rinsed with PBS and stained with crystal violet.

The experiments were performed 5 times in triplicates. Data are shown as the mean ± standard deviation. Statistical significance was determined using the student's *t* test.

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