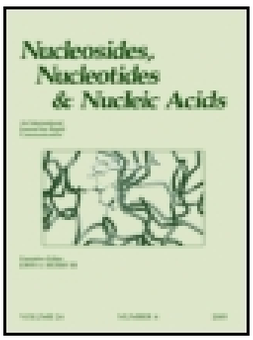


Nucleosides, Nucleotides and Nucleic Acids

ISSN: 1525-7770 (Print) 1532-2335 (Online) Journal homepage: http://www.tandfonline.com/loi/lncn20

N6-isopentenyladenosine a new potential anti-angiogenic compound that targets human microvascular endothelial cells *in vitro*

Sara Castiglioni, Valentina Romeo, Silvana Casati, Roberta Ottria, Cristiana Perrotta, Pierangela Ciuffreda & Jeanette A. M. Maier

To cite this article: Sara Castiglioni, Valentina Romeo, Silvana Casati, Roberta Ottria, Cristiana Perrotta, Pierangela Ciuffreda & Jeanette A. M. Maier (2018): N6-isopentenyladenosine a new potential anti-angiogenic compound that targets human microvascular endothelial cells *in vitro*, Nucleosides, Nucleotides and Nucleic Acids

To link to this article: https://doi.org/10.1080/15257770.2018.1503673

Published online: 22 Nov 2018.

Submit your article to this journal

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=lncn20

NUCLEOSIDES, NUCLEOTIDES AND NUCLEIC ACIDS
https://doi.org/10.1080/15257770.2018.1503673

Taylor & Francis
Taylor & Francis Group

Check for updates

N6-isopentenyladenosine a new potential anti-angiogenic compound that targets human microvascular endothelial cells *in vitro*

Sara Castiglioni, Valentina Romeo, Silvana Casati, Roberta Ottria, Cristiana Perrotta, Pierangela Ciuffreda, and Jeanette A. M. Maier

Dipartimento di Scienze Biomediche e Cliniche "L. Sacco", Università degli Studi di Milano, Milano, Italy

ABSTRACT

N6-isopentenyladenosine is an anti-proliferative and pro-apoptotic atypical nucleoside for normal and tumor cells. Considering the role of angiogenesis in various diseases, we investigated the cytotoxic effect of N6-isopentenyladenosine on human microvascular endothelial cells, protagonists in angiogenesis. Our results show that N6-isopentenyladenosine induced a significant reduction of cell viability, upregulated p21 and promoted caspase-3 cleavage in a dose dependent manner leading to apoptotic cell death as detected by FACS analysis. To understand structure-function relationship of N6-isopentenyladenosine, we investigated the effect of some N6-isopentenyladenosine analogs. Our results suggest that N6-isopentenyladenosine and some of its derivatives are potentially novel angiostatic agents and might be associated with other anti-angiogenic compounds for a better outcome.

ARTICLE HISTORY
Received 20 December 2017
Accepted 19 July 2018

KEYWORDS
N6-isopentenyladenosine; HMEC; angiogenesis

Introduction

N6-isopentenyladenosine (iPA, **1a**), an atypical nucleoside that belongs to the cytokinin family,[1] is found in transfer RNA (tRNA) of eukaryotic and prokaryotic cells.[2] In particular, in mammals iPA is part of tRNA[Ser]sec and the cognate tRNA-isopentenyltransferase (TRIT1) has been hypothesized to be a putative tumor suppressor.[3]

Several studies have demonstrated the anti-proliferative and pro-apoptotic effects of iPA and some analogs in a broad variety of normal diploid cells and in different tumor cell lines derived from carcinomas, sarcomas, and myeloma.[4–11]

In HeLa cells, iPA-induced apoptosis was associated with the inhibition of both AKT and transforming growth factor β–activated kinase 1 (TAK1) activities. Since iPA binds the RING domain of tumor necrosis factor

CONTACT Roberta Ottria roberta.ottria@unimi.it Dipartimento di Scienze Biomediche e Cliniche "L. Sacco", Università degli Studi di Milano, Milano 20157, Italy

© 2018 Taylor & Francis Group, LLC

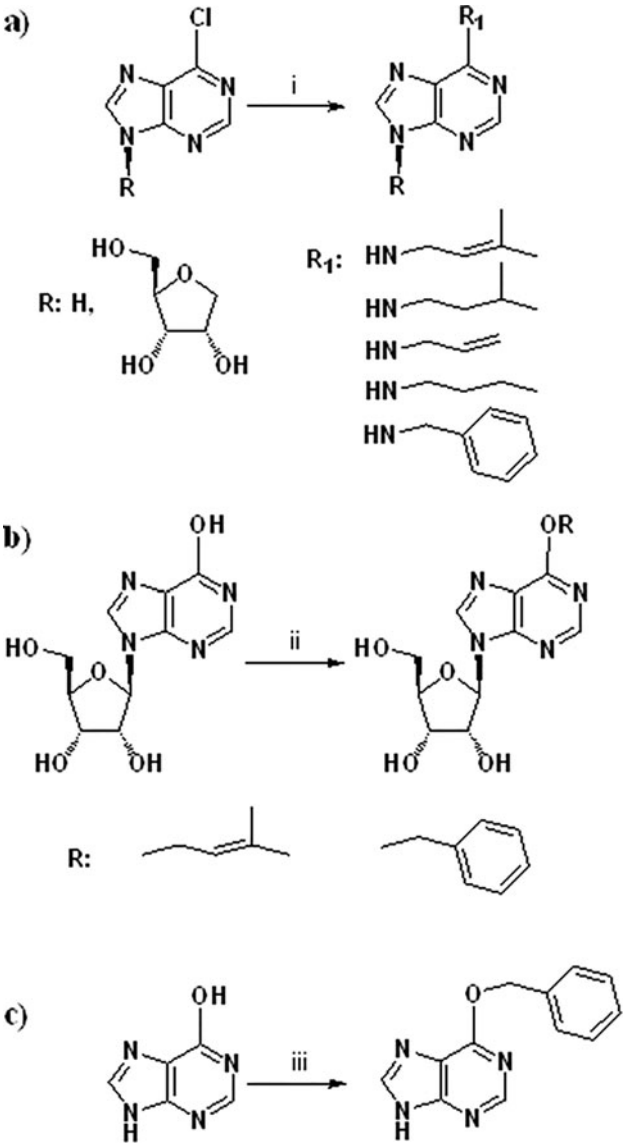
receptor–associated factor 6 (TRAF6), which, in turn, is involved in the ubiquitination and activation of AKT and TAK1 signaling pathways, the anti-proliferative effect of iPA in HeLa cells is probably due to its interaction with TRAF6.[12] In human endothelial cells derived from the umbilical vein (HUVEC) iPA significantly impaired the viability through the activation of AMP-kinase (AMPK).[13]

Endothelial cells are highly heterogeneous[14] as the results of the different mechanical and structural characteristics due to the different caliber, of the signals originating in diverse microenvironments and also of the different functions exerted by the endothelium in different tissues. HUVEC are widely considered as a model of macrovascular endothelium but do not reflect the highly specialized nature of microvascular endothelial cells, which are crucial in regulating tissue homeostasis and are protagonists in angiogenesis and inflammation. To test the potential anti-angiogenic activity of iPA and analogs, we utilized Human Microvascular Endothelial Cells (HMEC). We found that iPA and some of its analogs are cytotoxic and promote apoptosis, thus suggesting that they might be useful as anti-angiogenic molecules.

Materials and methods

Reagents

N6-isopentenyladenine (**3c**), trans-zeatin riboside (**4**), trans-zeatin (**5**), 6-chloropurine riboside, 6-chloropurine, all available starting amines, the other reagents and all solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). iPA (**1a**) and the other molecules were prepared and purified as described previously.[8,15,16] Briefly, compounds (**1a–e, 3b**) were synthesized as follow: to a solution of 6-chloropurine riboside or 6-chloropurine in absolute EtOH, Et_3N, and the appropriate amine 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 crystallized from MeOH (Scheme 1a).[15] For inosine derivatives (**2a,b**) $BaCO_3$ and 3,3-dimethylallylbromide or benzyl bromide were added to a solution of inosine in DMF and the mixture was stirred at room temperature for 24 h. 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 (Scheme 1b).[16] At last hypoxanthine derivative (**3a**) was synthesized in the following manner: to a suspension of sodium hydride in dry tetrahydrofuran, cooled with an ice bath, benzylalcohol was added slowly. After 20 min, 6-chloropurine was added and the solution refluxed for 24 h. After



Scheme 1. (**a**) (i) RNH_2, EtOH, Et_3N, r.t, 3 h; (**b**) (ii) RBr, $BaCO_3$, DMF, rt, 24 h; (**c**) (iii) benzylalcohol, THF dry, NaH, 0 °C 20 min, then 24 h reflux.

removal of the solvent under vacuum, the residue was dissolved in a 10% solution of NH_4Cl and extracted with dichloromethane and ethyl acetate, the crude was purified by colon chromatography (Scheme 1c).[8] Purity ($\geq$99%) and structure of all compounds were verified by HPLC, NMR, as described before,[17,18] and mass spectrometry measurements. Structures of all compounds are presented in Figure 1. Stock solutions of these compounds were prepared in DMSO (final concentration 10 mM) and kept at -20 °C. Appropriate dilutions were freshly prepared in culture medium just

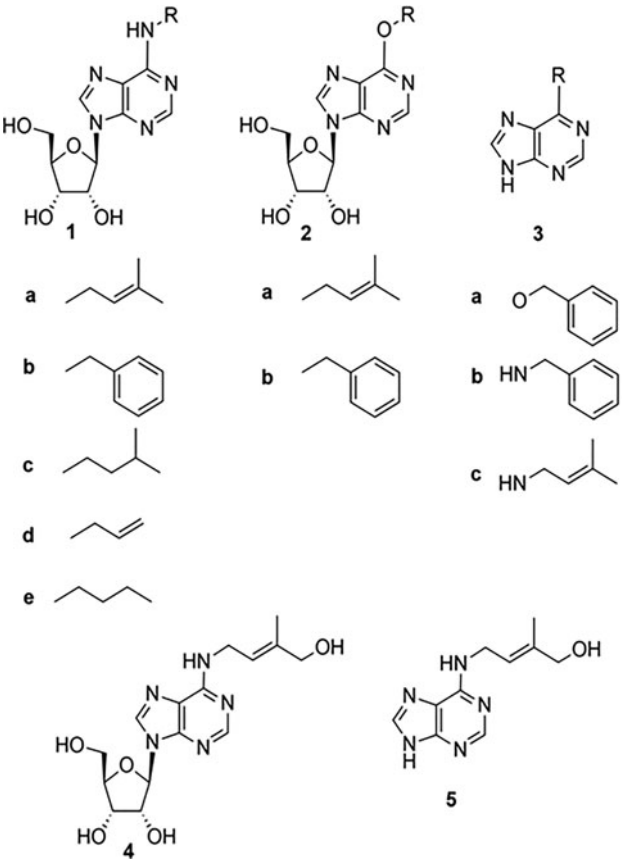


Figure 1. Structures of all the compounds used.

prior the assays. The controls were added with the final concentrations of DMSO (0.01%).

Cell culture

HMEC were obtained from Centers for Disease Control and Prevention (Atlanta, United States) and grown in MCDB131 (Invitrogen, Milan, Italy) containing Epidermal Growth Factor (EGF) (10 ng/mL), hydrocortisone (1 µg/mL) and 10% FBS on 2% gelatin-coated dishes.[19,20] To evaluate the effects of iPA and derivatives, MTT assay was used in HMEC seeded in 96 well/plates. Briefly, the medium was replaced with medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT, 0.5 mg/ml) (Sigma Aldrich, 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.[21] Absorbance was measured at 550 nm. In dose dependent experiments, HMEC were exposed to different concentrations of iPA, **1b** and **1c** and IC50 values were calculated by

non-linear regression analysis of Log[iPA or derivatives concentration]/cell viability using GraphPad Prism 5. In some experiments, HMEC were exposed also to human recombinant interferon (IFN)γ (PromoKine, Bio-Connect B.V., The Netherlands). Data represent the mean ± standard deviation of three separate experiments in triplicates.

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

HMEC cells were fixed in 70% ethanol for 30 min, centrifuged at 2000 rpm for 10 min and washed with ice-cold PBS. The pellets were resuspended in PBS containing 25 µg/ml propidium iodide (Sigma Aldrich), incubated in the dark at 37 °C for 30 min, and then analyzed using Coulter EPICS 753 flow cytometer (Beckman Coulter, Miami, FL).[21] The percentage of cells in each phase of the cell cycle was determined by using a Multicycle software version 2.53 (Phoenix Software, San Diego, CA).

Flow cytometric analysis of cell death

Phosphatidylserine exposure on the outer leaflet of the plasma membrane was detected by analysis of cells stained for 15 min with Alexa Fluor 488-labeled Annexin V (1 µg/ml) (Molecular Probes-Thermo Fisher Scientific, Waltham, MA, USA) and Propidium Iodide (Sigma-Aldrich, Saint Louis, MO, USA), using a Fluorescence-Activated Cell Sorter (Gallios, Beckman-Coulter, Brea, CA, USA) as described previously.[21]

Western blot analysis

HMEC cells were lysed, separated on SDS-PAGE, and transferred to nitrocellulose sheets. Western analysis was performed using antibodies against p21, actin (Santa Cruz Biotechnology, Dallas, Texas, USA) and caspase 3 (Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies were labeled with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The SuperSignal chemiluminescence kit (Pierce, Rockford, IL, USA) was used to detect immunoreactive proteins.

Statistical analysis

Statistical significance was determined using the Student's T test and set at p values less than 0.05. In the figures *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

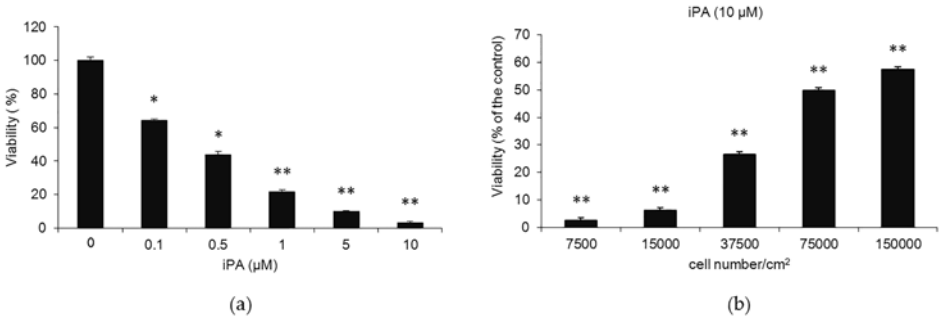


Figure 2. iPA induces cytotoxicity on HMEC cells. (**a**) HMEC were exposed to various concentrations of iPA for 72 h or (**b**) different cell densities were cultured in the presence of 10 μM of iPA for 72 h. In **a** and **b** cell viability was measured by MTT assay and expressed as the percentage of control. Data are represented as the means ± standard deviation of at least three separate experiments. *p* value was calculated *vs* untreated cells: $^{*}p < 0.05$, $^{**}p < 0.01$.

Results

iPA is cytotoxic for HMEC

We first studied the toxicity of iPA by culturing HMEC in a medium containing increasing concentrations (from 0.1 to 10 μM) of this cytokinin for 72 hours. Using the MTT assay we analyzed the effect of this molecule on cell vitality. iPA induces a significant dose dependent reduction of cell viability (Figure 2a). Cytotoxicity was statistically significant at a concentration of 0.1 μM with an Inhibitory Concentration 50 (IC50) = 0.5 ± 0.2 μM.

Since 10 μM is cytotoxic for most neoplastic cells,[7–9] we studied the effect of this concentration of iPA in HMEC seeded at different densities (from 7500 to 150000 cells/cm^2). Indeed, under physiologic conditions the endothelial cells form a confluent monolayer, but in tumor angiogenesis the morphology of neo-formed vessels is chaotic and it is possible that the endothelium is discontinuous or also absent.[22] On these bases, we performed an MTT assay in sparse and confluent cells and found that HMEC were more sensitive to the toxic effect of iPA when seeded at low density (Figure 2b).

iPA induces cell cycle arrest and apoptosis

To understand the mechanisms of iPA-induced cytotoxicity, HMEC were cultured in the presence of iPA (10 μM) for 24 h and cell cycle distribution was analyzed by FACS. As shown in Figure 3a, iPA induced an accumulation of HMEC in G1 phase. This finding paralleled with the increase of the G1/S-CDK blocker and DNA synthesis inhibitor p21 as detected by western blot (Figure 3b). Figure 3a also revealed the decrease of the cells in the S and G2/M phases. The most significant result was the increase of the percentage of the sub-G1 cell population in iPA-treated vs untreated cells

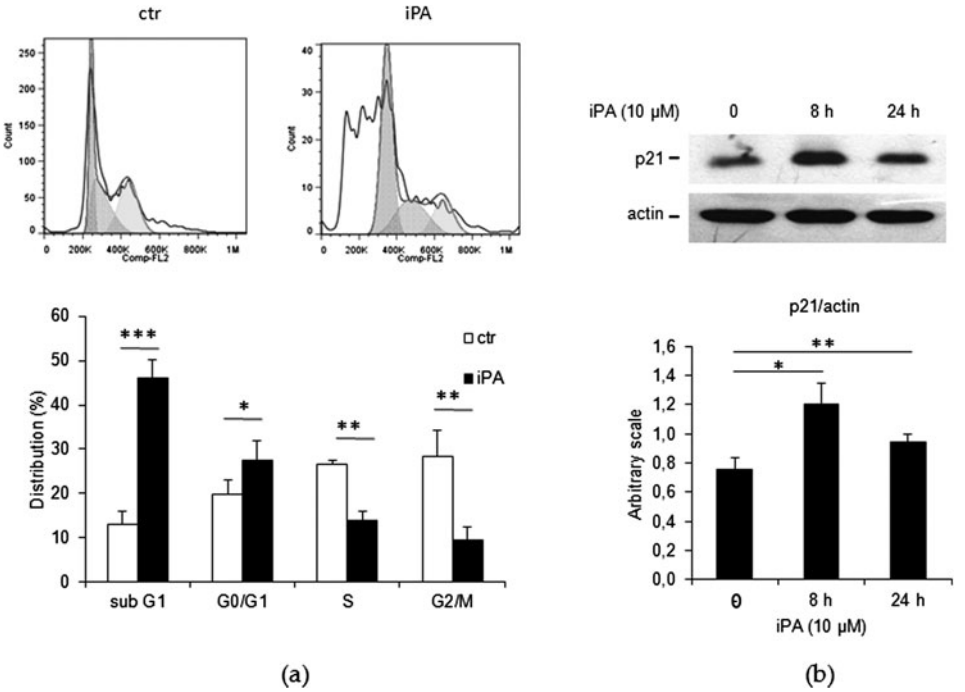


Figure 3. iPA induces cell cycle arrest. (**a**) HMEC exposed to iPA (10 μM) for 24 h were analyzed by FACS to study the cell cycle distribution compared to untreated cells. The data are shown as the percentage of cells in sub-G1, G1, S and G2/M phases. Each bar is the mean of three separate experiments in triplicate ± standard deviation. (**b**) HMEC were exposed to iPA (10 μM) for 8 and 24 h. Cells extracts (100 μg/lane) were processed for western blot using antibodies against p21. Actin is used as control of loading. Densitometric analysis was performed by the ImageJ software and p21/actin ratio was calculated on three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(from 13% to 43%). The sub-G1 peak in the histogram identifies the relative amount of apoptotic cells. Using Annexin V/PI staining we confirmed a marked increase of the apoptotic cell population in iPA treated cells *vs* controls (Figure 4a). In particular, about 29 and 50% of 1 and 10 μM iPA treated cells resulted apoptotic (Annexin V positive and PI negative cells + Annexin V and PI positive cells). This finding prompted us to investigate the processing of caspase-3, which is a critical executioner of apoptosis. Indeed, caspase-3 is activated by proteolytic cleavage from an inactive zymogen into activated p17 and p19 fragments. Western blot shows that 24 h exposure to iPA (1 and 10 μM) induced caspase-3 cleavage in a dose-dependent fashion (Figure 4b).

Cytotoxic effect of iPA derivatives

To better understand the relationship between iPA structure and its biological activity, we treated HMEC with some iPA derivatives to compare

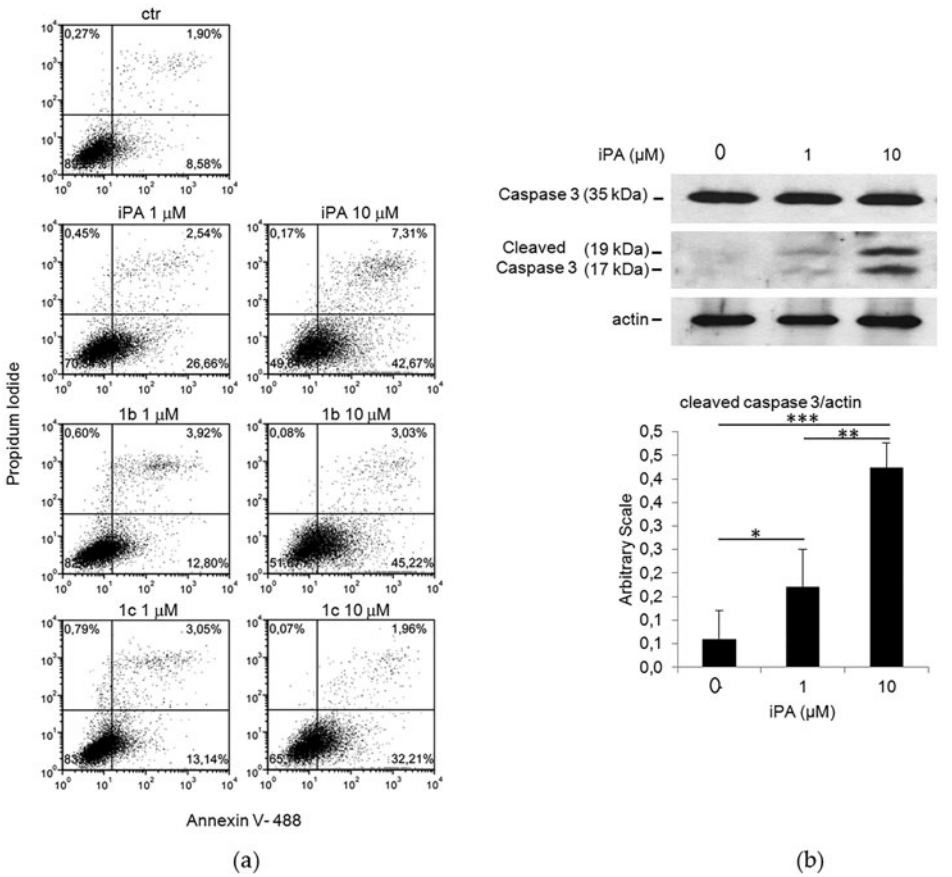


Figure 4. iPA induces apoptosis. HMEC were exposed to iPA (1 and 10 µM) for 24 h. (**a**) Annexin V/PI staining was performed and the cells were analyzed by flow cytometry. A representative experiment is shown. (**b**) Cells extracts (100 µg/lane) were processed for western blot using antibodies against cleaved caspase 3. Actin and total caspase 3 are used as control of loading. Densitometric analysis was performed by the ImageJ software and cleaved caspase 3/actin ratio was calculated on three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

their cytotoxic effect. Compounds reported in Figure 1 have been selected as the more active molecules from a library of iPA derivatives synthesized and assessed for their biological activity on cancer cells in our laboratories.[8,9,15,16] On the basis of the results obtained with iPA (Figure 2), the different compounds were assessed on sparse and confluent HMEC. By MTT assay, we show that some compounds (**1b, 1c, 1d, 1e**, **4**) exert a significant toxic effect that is dependent on HMEC density (Figure 5a). Indeed, sparse HMEC cells resulted more sensitive to these compounds. We selected compounds **1b** and **1c**, which induced 80 and 50% reduction of cell viability on sparse HMEC, respectively, for further studies. HMEC were exposed to different doses of **1b** and **1c**, from 0.1 to 10 µM. Our results demonstrate that the reduction of cell viability was dose dependent

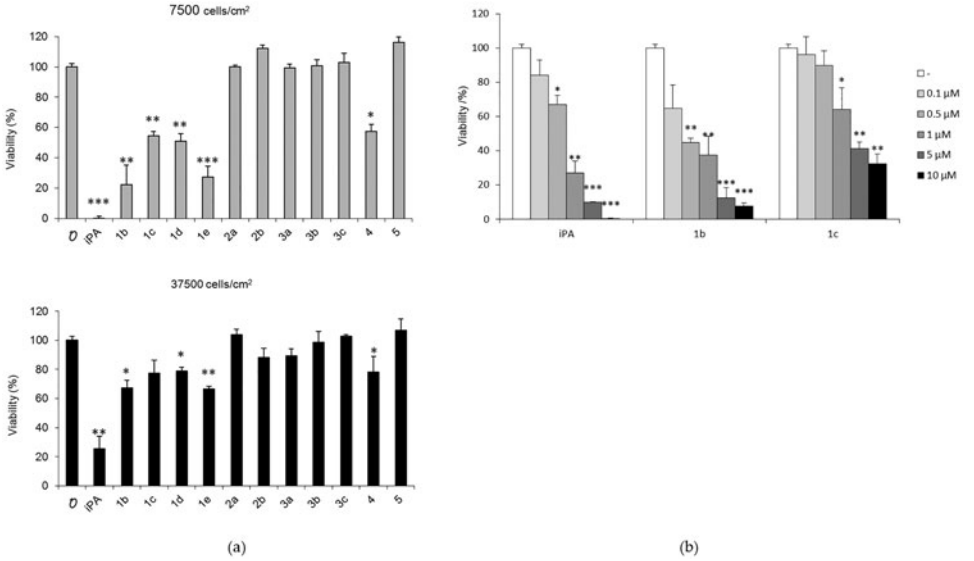


Figure 5. Cytotoxic effect of some iPA derivatives. (**a**) Sparse (7500 cells/cm^2) and confluent (37500 cells/cm^2) HMEC were cultured for 72 h in the presence of iPA. Viable cells were evaluated by MTT assay. (**b**) MTT assay was performed on HMEC cultured in the presence of different doses of iPA and its derivatives **1b** and **1c**.

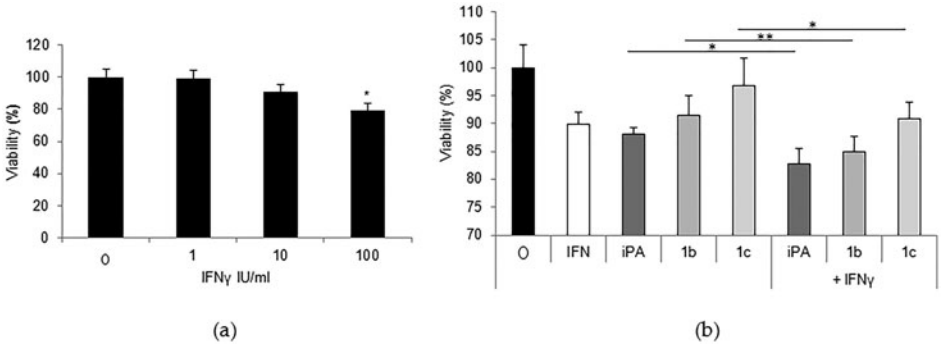


Figure 6. The additive effect of iPA and its derivatives with IFNγ. (**a**) HMEC were treated with IFNγ (1, 10, 100 IU/ml) and (**b**) with iPA and its derivatives **1b** and **1c** (1 µM) with or without IFNγ for 72 h. In **a** and **b** cell viability was measured by MTT assay and expressed as the percentage of control. Data are represented as the means ± standard deviation of at least three separate experiments. $^*p < 0.05$, $^{**}p < 0.01$.

(Figure 5b) with an IC50 of 0.4 ± 0.1 µM for **1b** and 3.5 ± 0.2 µM for **1c**. Moreover, using Annexin V/PI staining we show that 17 and 48% of 1 and 10 µM **1b** treated cells or 16 and 34% of 1 and 10 µM **1c** treated cells were apoptotic (Figure 4a)

The effect of iPA and its derivatives in association with IFNγ

We also investigated the cytotoxicity of iPA and its derivatives added in combination to the culture medium of HMEC. Indeed, among its multiple

functions, IFNγ inhibits endothelial growth and migration.[23] Initially, we studied HMEC sensitivity to IFNγ. IFNγ significantly impairs HMEC viability at a concentration of 100 IU/ml (Figure 6a). Using this concentration we demonstrate a significant additive effect with iPA 1 μM and its derivatives **1b** and **1c** (Figure 6b).

Discussion

In vivo microvascular endothelial cells are quiescent with a turnover rate of approximately once every three years.[24] In certain conditions, these quiescent cells are activated and initiate angiogenesis, i.e. the formation of new vessels from preexisting ones. Angiogenesis is fundamental in physiological processes, from embryonic development to wound repair.[25] However, excessive angiogenesis contributes to the pathogenesis of various diseases, among which cancer, blinding retinopathies, psoriasis, arthritis, endometriosis and Crohn's disease.[25] Also virus and bacteria carry angiogenic genes or upregulate angiogenic genes in the host.[26] Several antiangiogenic drugs with various indications are available or in various phases of clinical trials, but there is constant need of new molecules that target microvascular endothelial cells. Considering the importance of angiogenesis for tumor growth and progression and the significant anti-proliferative activity of iPA on a large number of tumor models, we analyzed iPA effects on human microvascular endothelial cells and found that it induces a dose dependent reduction of their viability. This is particularly evident in sparse cells. It is noteworthy that HMEC are very sensitive to the cytotoxic effect of iPA, with an IC50 = 0.5 μM, while tumor cells usually require higher concentrations. For instance, iPA toxic effect in bladder cancer T24 cells is significant at 1 μM, with an IC50 around 3 μM.[8] As previously shown in T24 cells,[8] also in HMEC, iPA promotes apoptosis as detected by FACS. In agreement with these results, we observed dose dependent caspase-3 cleavage. Interestingly, p21, which is considered a marker of endothelial senescence,[27] is upregulated by iPA. It is noteworthy that senescent endothelial cells are more susceptible to apoptosis than their young counterparts because of the reduced activity of the anti-apoptotic endothelial nitric oxide synthase (eNOS).[28] Therefore, we hypothesize that the induction of apoptosis might take place in two steps. Some cells immediately undergo apoptosis, others acquire a senescent phenotype that sensitizes them to pro-apoptotic stimuli.

We also studied the toxic effects of a series of iPA derivatives with modification of the alkylation in N6-position and of the base moiety. HMEC were exposed for 72 h to 11 different iPA derivatives and cell viability was analyzed. Only five of the N6-substituted adenosine analogs tested affected

cell viability, while the other modifications of iPA structure abolished iPA biological activity. In particular, compounds **1b** and **1c** induce apoptosis in a dose dependent fashion as detected by FACS analysis after Annexin V/PI staining. The active compounds are characterized by side chains containing at least three carbon atoms and the presence of an unsaturation in the chain. However, it is important to underscore that the five active compounds are less cytotoxic than iPA.

It is noteworthy that iPA and its derivatives **1b** and **1c** show an additive effect with IFNγ, which is known to inhibit endothelial migration and growth, two crucial events in angiogenesis.[23] Our results suggest that iPA and derivatives might be used in association with iPA to obtain a more effective inhibition of endothelial cell proliferation.

On these bases, we conclude that iPA and some of its derivatives might be interesting in an oncologic setting, since they hit both neoplastic and microvascular endothelial cells. They might also be utilized in any other condition characterized by excessive angiogenesis.

Acknowledgements and Funding

This work was partially supported by: "Università di Milano, Linea 2 – Azione A 2016-2017".

Disclosure statement

No potential conflict of interest was reported by the author(s).

References

[1] Faust, J. R.; Dice, J. F. Evidence for Isopentenyladenine Modification on a Cell Cycle-Regulated Protein. *J. Biol. Chem* **1991**, *266*, 9961–9970.

[2] Kersten, H. On the Biological Significance of Modified Nucleosides in tRNA. *Prog. Nucleic Acid Res. Mol. Biol.* **1984**, *31*, 59–114.

[3] Spinola, M.; Galvan, A.; Pignatiello, C.; Conti, B.; Pastorino, U.; Nicander, B.; Paroni, R.; Dragani, T. A. Identification and Functional Characterization of the Candidate Tumor Suppressor Gene TRIT1 in Human Lung Cancer. *Oncogene* **2005**, *24*, 5502–5509.

[4] Bifulco, M.; Malfitano, A. M.; Proto, M. C.; Santoro, A.; Caruso, M. G.; Laezza, C. Biological and Pharmacological Roles of N6-Isopentenyladenosine: An Emerging Anticancer Drug. *Anticancer Agents Med Chem.* **2008**, *8*, 200–2004.

[5] Spinola, M.; Colombo, F.; Falvella, F. S.; Dragani, T. A. N6-Isopentenyladenosine: A Potential Therapeutic Agent for a Variety of Epithelial Cancers. *Int. J. Cancer*. **2007**, *120*, 2744–2748.

[6] Ishii, Y.; Hori, Y.; Sakai, S.; Honma, Y. Control of Differentiation and Apoptosis of Human Myeloid Leukemia Cells by Cytokinins and Cytokinin Nucleosides, Plant Redifferentiation-Inducing Hormones. *Cell. Growth Differ* **2002**, *13*, 19–26.

[7] Castiglioni, S.; Casati, S.; Ottria, R.; Ciuffreda, P.; Maier, J. A. N6-Isopentenyladenosine and Its Analogue N6-Benzyladenosine Induce Cell Cycle Arrest and Apoptosis in Bladder Carcinoma T24 Cells. *Anticancer Agents Med Chem*. **2013**, *13*, 672–678.

[8] Casati, S.; Ottria, R.; Baldoli, E.; Lopez, E.; J. A. M.; Ciuffreda, P. Effects of Cytokinins, Cytokinin Ribosides and Their Analogs on the Viability of Normal and Neoplastic Human Cells. *Anticancer Res* **2011**, *31*, 3401–3406.

[9] Ottria, R.; Casati, S.; Maier, J. A. M.; Mariotti, M. Ciuffreda P. Novel Isopentenyladenosine Analogues: Synthesis, Characterization, and Evaluation of Antiproliferative Activity on Bladder Carcinoma Cells. *Nucleos. Nucleot. Nucl* **2009**, *28*, 736–751.

[10] Ranieri, R.; Ciaglia, E.; Amodio, G.; Picardi, P.; Proto, M. C.; Gazzerro, P.; Laezza, C.; Remondelli, P.; Bifulco, M.; Pisanti, S. N6-Isopentenyladenosine Dual Targeting of AMPK and Rab7 Prenylation Inhibits Melanoma Growth through the Impairment of Autophagic Flux. *Cell Death Differ*. **2018**, *25*, 353–367.

[11] Ciaglia, E.; Grimaldi, M.; Abate, M.; Scrima, M.; Rodriquez, M.; Laezza, C.; Ranieri, R.; Pisanti, S.; Ciuffreda, P.; Manera, C.; et al. The Isoprenoid Derivative N6-Benzyladenosine CM223 Exerts Antitumor Effects in Glioma Patient-Derived Primary Cells through the Mevalonate Pathway. *Br. J. Pharm* **2017**, *174*, 2287–2301.

[12] Li, M.; Qi, Y.; Wei, J.; Lu, L.; Zhao, X.; Zhou, L. N6-Isopentenyladenosine Promoted HeLa Cell Apoptosis through Inhibitions of AKT and Transforming Growth Factor β-Activated Kinase 1 Activation. *Tumour. Biol.* **2017**, *39*, 101042831769596.

[13] Pisanti, S.; Picardi, P.; Ciaglia, E.; Margarucci, L.; Ronca, R.; Giacomini, A.; Malfitano, A. M.; Casapullo, A.; Laezza, C.; Gazzerro, P.; Bifulco, M. Antiangiogenic Effects of N6-Isopentenyladenosine, an Endogenous Isoprenoid End Product, Mediated by AMPK Activation. *Faseb J* **2014**, *28*, 1132–1144.

[14] Aird, W. C. Endothelial Cell Heterogeneity. *Cold Spring Harb Perspect Med* **2012**, *2*, a006429

[15] Ottria, R.; Casati, S.; Baldoli, E.; Maier, J. A. M.; Ciuffreda, P. N^6-Alkyladenosines: synthesis and Evaluation of *in Vitro* Anticancer Activity. *Bioorg. Med. Chem* **2010**, *18*, 8396–8402.

[16] Ottria, R.; Casati, S.; Manzocchi, A.; Baldoli, E.; Mariotti, M.; Maier, J. A. M.; Ciuffreda, P. Synthesis and Evaluation of in Vitro Anticancer Activity of Some Novel Isopentenyladenosine Derivatives. *Bioorg. Med. Chem* **2010**, *18*, 4249–4254.

[17] Casati, S.; Manzocchi, A.; Ottria, R.; Ciuffreda, P. 1H, 13C and 15N NMR Spectral Assignments of Adenosine Derivatives with Different Amino Substituents at C^6position. *Magn. Reson. Chem*. **2011**, *49*, 279–283.

[18] Casati, S.; Manzocchi, A.; Ottria, R.; Ciuffreda, P. 1H, 13C and 15N NMR Assignments for N6-isopentenyladenosine/inosine analogues . *Magn. Reson. Chem*. **2010**, *48*, 745–748.

[19] Castiglioni, S.; Caspani, C.; Cazzaniga, A.; Maier, J. A. Short- and Long-Term Effects of Silver Nanoparticles on Human Microvascular Endothelial Cells. *World J. Biol. Chem*. **2014**, *5*, 457–464.

[20] Castiglioni, S.; Cazzaniga, A.; Maier, J. A. Potential Interplay between NFκB and PPARγ in Human Dermal Microvascular Endothelial Cells Cultured in Low Magnesium. *Magnes. Res* **2014**, *27*, 86–93.

[21] Castiglioni, S.; Cazzaniga, A.; Perrotta, C.; Maier, J. A. Silver Nanoparticles-Induced Cytotoxicity Requires ERK Activation in Human Bladder Carcinoma Cells. *Toxicol. Lett*. **2015**, *237*, 237–243.

[22] Reitan, N. K.; Thuen, M.; Goa, P. E.; de Lange Davies, C. Characterization of Tumor Microvascular Structure and Permeability: Comparison between Magnetic Resonance Imaging and Intravital Confocal Imaging. *J. Biomed. Opt*. **2010**, *15*, 036004.

[23] Maier, J. A.; Morelli, D.; Lazzerini, D.; Mènard, S.; Colnaghi, M. I.; Balsari, A. Inhibition of Fibronectin-Activated Migration of Microvascular Endothelial Cells by Interleukin-1alpha, Tumour Necrosis Factor Alpha and Interferon Gamma. *Cytokine* **1999**, *11*, 134–139.

[24] Foreman, K. E.; Tang, J. Molecular Mechanisms of Replicative Senescence in Endothelial Cells. *Exp. Gerontol*. **2003**, *38*, 1251–1257.

[25] Tahergorabi, Z.; Khazaei, M. A Review on Angiogenesis and Its Assays. *Iran J Basic Med. Sci*. **2012**, *15*, 1110–1126.

[26] Harada, K.; Lu, S.; Chisholm, D. M.; Syrjänen, S.; Schor, A. M. Angiogenesis and Vasodilation in Skin Warts. Association with HPV Infection. *Anticancer Res*. **2000**, *20*, 4519–4523.

[27] Bernardini, D.; Ballabio, E.; Mariotti, M.; Maier, J. A. Differential Expression of EDF-1 and Endothelial Nitric Oxide Synthase by Proliferating, Quiescent and Senescent Microvascular Endothelial Cells. *Biochim. Biophys. Acta* **2005**, *1745*, 265–272.

[28] Rippe, C.; Blimline, M.; Magerko, K. A.; Lawson, B. R.; LaRocca, T. J.; Donato, A. J.; Seals, D. R. MicroRNA Changes in Human Arterial Endothelial Cells with Senescence: relation to Apoptosis, eNOS and Inflammation. *Exp. Gerontol* **2012**, *47*, 45–51.