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**NOVEL INSIGHTS INTO THE BIOLOGICAL EFFECTS OF THE
ISOPRENOID DERIVATIVE
N6-ISOPENTENYLADENOSINE:
INVOLVEMENT OF THE METABOLIC SENSOR AMPK IN
ANGIOGENESIS INHIBITION**

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ABSTRACT

N6-isopentenyladenosine (iPA) is a modified adenosine characterized by an isopentenyl chain derived by dimethylallyl pyrophosphate (DMAPP), an intermediate of the metabolic pathway of mevalonate, that is known to be deregulated in cancer.

iPA is an endogenous isoprenoid-derived product present in mammalian cells as a free nucleoside in the cytoplasm, or in a tRNA-bound form, displaying well established pleiotropic biological effects, including a direct anti-tumor activity against several cancers. However, the precise mechanism of action of iPA in inhibiting cancer cell proliferation remains to be clarified.

In this work, we investigated whether iPA could directly interfere with the angiogenic process, fundamental to cancer growth and progression, and if the growth and proliferation of human melanoma cells, known for their highly angiogenic phenotype, could be affected by the treatment with iPA. Finally, we investigated if iPA could have an immunomodulatory role targeting directly human natural killer (NK) cells, components of innate immunity that participate in immunity against neoplastic cells, in order to provide a cooperative and multifactorial mode of action of iPA to arrest cancer growth. To evaluate the potential involvement of iPA in angiogenesis, we employed human umbilical vein endothelial cells (HUVECs) as a suitable *in vitro* model of angiogenesis, by evaluating the viability, proliferation, migration, invasion, tube formation, and molecular mechanisms involved. Data were corroborated in mice by using a gel plug assay. iPA dose- and time-dependently inhibited all the neoangiogenesis stages, with an IC₅₀ of 0.98 μ M. We demonstrated for the first time that iPA was monophosphorylated into iPA 5'-monophosphate (iPAMP) by adenosine kinase (ADK) inside the cells. iPAMP is the active form that inhibits angiogenesis through the direct activation of AMP-kinase (AMPK). Indeed, all effects were completely reversed by pre-treatment with 5-iodotubercidin (5-Itu), an ADK inhibitor. The isoprenoid intermediate isopentenyl pyrophosphate (IPP), which shares the isopentenyl moiety with iPA, was ineffective in the inhibition of angiogenesis, thus showing that the iPA structure is specific for the observed effects. Thus, iPA is a novel AMPK activator and could represent a useful tool for the treatment of diseases where excessive neoangiogenesis is the underlying pathology.

The activation of AMPK seems to be the mechanism by which iPA exerts also its anticancer effects in A375 human melanoma cells. Indeed, in order to evaluate if iPA could be a useful agent able to inhibit tumor angiogenesis, we tested *in vitro* the ability of iPA to arrest the proliferation and the growth of melanoma, a tumor known for its highly angiogenic phenotype. In particular, we performed co-cultures of HUVEC and A375 cells, and we evaluated melanoma cells vitality to elucidate their cell fate following iPA-treatment. Moreover, the molecular mechanism elicited by

iPA in this cancer model was analyzed. According with endothelial cells, iPA (from 2.5 to 10 μM) was able to inhibit melanoma cell growth, inducing autophagy and subsequently apoptosis, through AMPK activation, acting as an AMP mimetic.

In the last part of this work, we analyzed a possible role of iPA in immune regulation. In analogy to the unique specificity for phosphoantigens like IPP, shown by human V γ 9V δ 2 T cells, we report for the first time the ability of iPA to selectively expand and directly target human NK cells. Interestingly, at submicromolar concentrations, iPA stimulated resting human NK cells and synergized with IL-2 to induce a robust *ex vivo* activation with significant secretion of CCL5 and CCL3 and a large increase in TNF- α and IFN- γ production when compared with IL-2 single cytokine treatment. Moreover, iPA was able to promote NK cell proliferation, upregulating the expression of specific NK cell activating receptors, as well as CD69 and CD107a expression. Cytotoxic activity of NK cells against tumor targets was due to a selective potent activation of MAPK signaling intermediaries downstream IL-2 receptor. The effect resulted at least in part from the fine modulation of the farnesyl diphosphate synthase (FDPS) activity, the same enzyme implicated in the stimulation of the human $\gamma\delta$ T cells. The iPA-driven modulation of FDPS can cause an enhancement of post-translational prenylation essential for the biological activity of key proteins in NK signaling and effector functions, such as Ras. These unanticipated properties of iPA provide additional piece of evidence of the immunoregulatory role of the intermediates of the mevalonate pathway and open novel therapeutic perspectives for this molecule as an immunomodulatory drug.

Taken together, these results highlight a synergic mode of action of iPA as antitumor agent, interfering with neovascularization and hence blood supply to nourish cancer cells, inducing autophagy and apoptosis directly in tumor cells and stimulating the immune response to attack neoplastic cells.