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N^6-Isopentenyladenosine, an isoprenoid cytokinin endowed with biological activity: cytotoxicity against MDA-MB-231 breast cancer cell line and interaction with bovine serum albumin

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Abstract: N^6-isopentenyladenosine (iPA) is a member of the cytokinin family of plant hormones that regulate plant cell growth and differentiation and is present in mammalian cells in a free form as a mononucleotide in cytoplasm or tRNA-bound. The cytotoxicity effect of iPA on MDA-MB-231 cells was measured by the SRB assay and the results showed that iPA could inhibit cell growth in a dose- and time-dependent manner with an IC_{50} value of 6.2 µmol/L at 72 h after the drug was added into the culture. The interaction of BSA with iPA was studied in aqueous solution at physiological conditions, using constant protein concentration of 10^{-5} M and various drug contents of 10^{-5} M – 2.5 x 10^{-7} M. UV-vis spectroscopic method was used to determine iPA binding mode, the binding constant and the effects of iPA complexation on protein secondary structure. The spectroscopic results showed that iPA is located along the polypeptide chains with overall affinity constant of K_{iPA}=4.9 x 10^4 M^{-1}.

Keywords: Isopentenyladenosine(iPA); MDA-MB-231; cytotoxicity; SRB assay; Bovine Serum Albumin (BSA).

Introduction

N^6-Isopentenyladenosine (iPA, Fig. 1) is a modified nucleoside found in transfer RNA (tRNA) of many eukaryotic and prokaryotic cells and is the only known cytokinin existing in animal tRNAs at a site that suggests a role in the process of transduction [1]. Many biological effects, both in plants and animal systems, also including antitumor effects on human and murine cells, have been attributed to iPA [2].

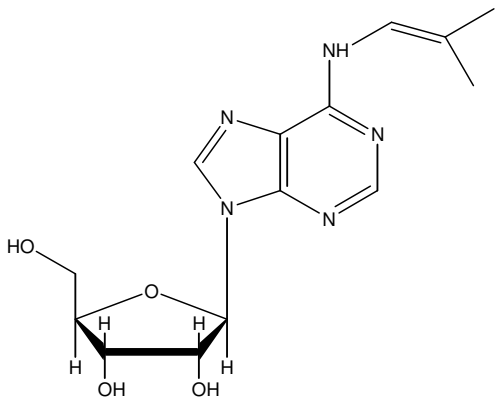

Fig. 1: Structure of N^6-Isopentenyladenosine (iPA)

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Nearly forty years ago, Gallo and co-workers[3] found that iPA exert a promoting or inhibitory effect on human cell growth, on the bases of used concentration and the cell cycle phase.

Very recently, iPA cytotoxicity has been thoroughly investigated by a few authors and results have confirmed the antiproliferative activity of iPA against several cancer cell lines [4-8]. We report here our studies results related to a cytotoxicity study of iPA against a specific breast cancer cell line (MDA-MB-231). Additionally, we present results obtained from the interaction of iPA and bovine serum albumin (BSA), as a model of the analogous human protein (HSA) one, to which with whom BSA shows a high homology with BSA [9]. This BSA is the most abundant protein constituent of the blood plasma, and that facilitates the disposition and transport of various exogenous and endogenous ligands to particular biotargets [10-12]. Since protein–drug binding greatly influences absorption, distribution, metabolism, and excretion properties of typical drugs [13], studies on the protein–drug binding are important for the elucidation of the reaction mechanisms, providing a pathway to the pharmacokinetics and pharmacodynamic mechanisms of these substances in various tissues.

Results and Discussion

Cytotoxic evaluation in vitro

In the presence of different doses of iPA, the cells were inhibited to a greater extent ranging from 10% to 90% with a loss of viable cells (Fig. 2). iPA dramatically inhibited the proliferation of MDAMB-231 cells in a concentration and time-dependent manner. The concentration which causes 50% inhibition of cell viability (IC50) by iPA was about 6.2 µmol/L at 72 h by employing the Sulforhodamine B (SRB) assay (Fig. 3).

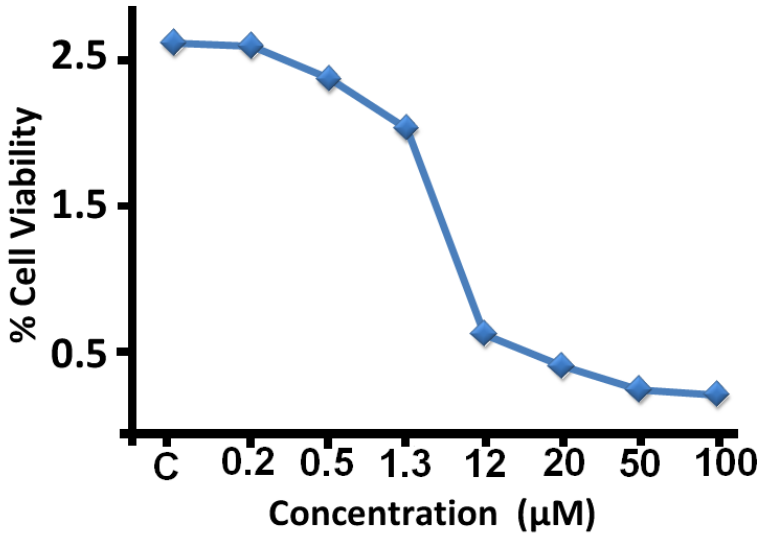

Fig. 2: Effects of iPA on the proliferation of MDA-MB-231. Dose of compound required to inhibit cell growth by 50% compared to untreated cell controls. All experiments were carried out in triplicate wells and each experiment was repeated twice.

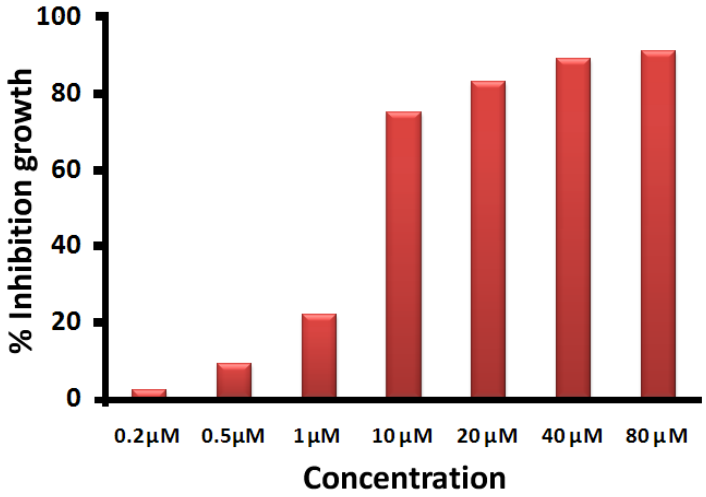

Fig. 3: Inhibition growth of of iPA on MDA-MB-231. The percentage of growth inhibition was calculated by using the equation: (1- At/Ac) x 100, where At and Ac represent the absorbance in treated and control cultures, respectively. IC_{50} was determined by interpolation from dose–response curves.

Assessment of cell shape and cell morphology

It is now generally accepted that cell death is an important phenomenon, reflected by the appearance of numerous publications on the subject every year. For more than 150 years, morphological features played the leading role in the description of cell death [14]. However, during the past three decades cell death has been characterized on the molecular level, which markedly increased our understanding of the morphology [15]. Under inverted microscope, cell shape and its changes can be observed clearly. Treated and untreated (control) cells were viewed using an inverted phase-contrast microscope model Zeiss and photographed using a Nikon camera attached to the microscope. Fig. 4 shows the incubation of the cells with different concentrations of iPA after 48 h treatment. Control group showed regular polygonal shape and cell antennas were short and there were very few round cells. Cells treated with iPA for two days showed obvious morphological changes, including the loss of adhesion, rounding, cell shrinkage and detachment from the substratum.

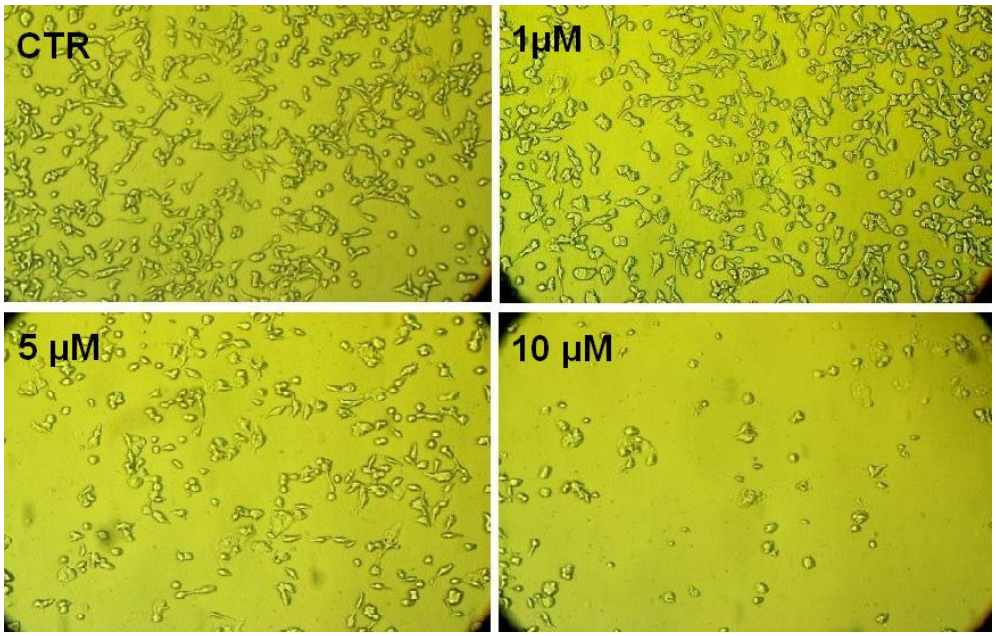

Fig. 4: Morphological analysis for the effects of iPA on MDA-MB-231 after 48 h incubation.

Binding Study of iPA with BSA

Conformational changes of native BSA after complexation with iPA

To explore the structural changes of BSA on addition of iPA, UV–vis absorbance spectra of BSA were measured at different concentrations of iPA (Fig. 5).

UV–vis spectra of BSA indicated that on addition of iPA, the absorption peaks in the visible region showed a moderate blue-shift. This indicated that with iPA, the peptide strands of the BSA molecules extended moderately and the hydrophobicity decreased [16].

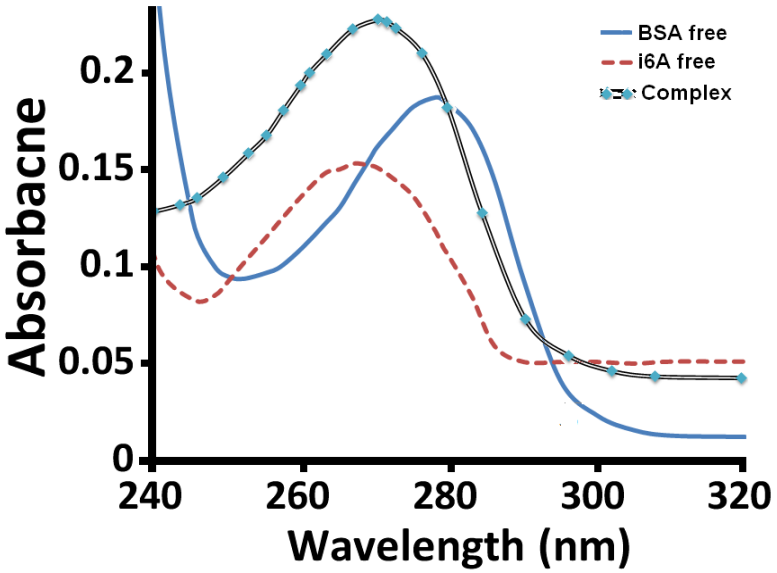

Fig. 5: UV-vis absorbance spectra of BSA in the presence of iPA.

Binding Constant of iPA with BSA

The iPA–BSA binding constant was determined using UV absorption spectroscopy [17,18] and the following equations can be established [19].

$$\text{BSA} + \text{iPA} \leftrightarrow \text{BSA} : \text{iPA}\ K_1 =$$

$$[\text{BSA} : \text{iPA}]/\{[\text{BSA}][\ \text{iPA}]\}$$

$$\text{BSA} : \text{iPA} + \text{iPA} \leftrightarrow \text{BSA} : \text{iPA}_2\ K_2 =$$

$$[\text{BSA} : \text{iPA}_2]/\ \{[\ \text{BSA} : \text{iPA}]\ [\text{iPA}]\}$$

$$\text{BSA} : \text{iPA}_2 + \text{iPA} \leftrightarrow \text{BSA} : \text{iPA}_3\ K_3 =$$

$$[\text{BSA} : \text{iPA}_3]/\ \{[\ \text{BSA: iPA}_3]\ [\text{iPA}]\}$$

$$\text{BSA} : \text{iPA}_3\ \text{iPA} + \text{iPA} \leftrightarrow \text{BSA} : \text{iPA}_4$$

$$K_4 = [\ \text{BSA} : \text{iPA}_4\ ]/\ \{[\ \text{BSA} : \text{iPA}_3]\ [\text{iPA}]\}$$

$$\text{Overall}\ K = K_1\ K_2\ K_3\ K_4 =$$

$$[\text{BSA: iPA}_4]/\ [\text{BSA}]\ [\text{iPA}]^4$$

The double reciprocal plot of 1/ (A-A0) vs. 1/(L) is linear and the binding constant (K) can be estimated from the ratio of the intercept to the slope (Fig. 6.) A0 is the initial absorption of the free BSA at 279 nm and A is the recorded absorption at different ipA concentrations (L). The overall binding constant K for ipA-BSA complexes is estimated to be 4.9 x 104 M-1. The value obtained is indicative of a good ipA-protein interaction.20 The reason for the low stability of the iPA-BSA complexes can be attributed to the presence of mainly hydrogen bonding interaction between protein donor atoms and the iPA polar groups or an indirect drug-protein interaction through water molecules.

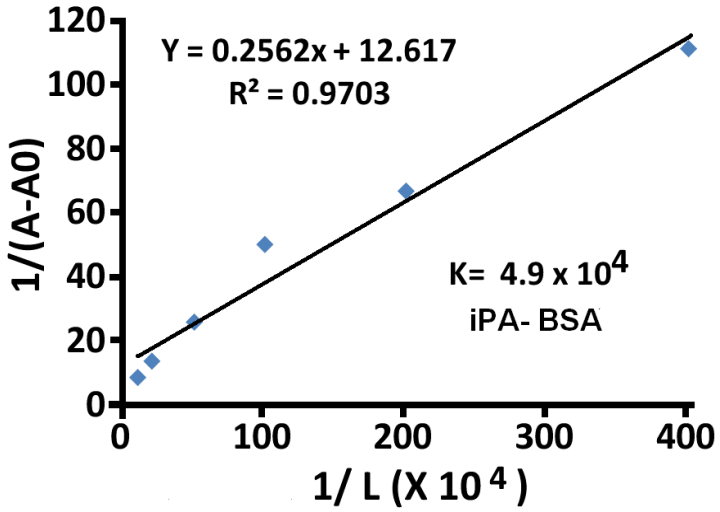

Fig. 6: The plot of 1/(A-A0) vs 1/L for BSA and iPA complexes where A_0 is the initial protein absorption band (279 nm) and A is the recorded absorption at different drug concentrations (L).

Conclusion

The present study demonstrates the anti-proliferative properties of iPA toward MDA-MB-231 human breast cancer cells. Anticancer activity of iPA was obtained after 72 h of exposure, using a standard SRB assay with an IC50 value of 6.2 μM. Our results indicate that iPA treatment of MDA-MB-231 cells causes morphological changes typical for apoptosis, such as loss of adhesion, rounding, cell shrinkage and detachment from the substratum. Interaction between iPA with BSA was also studied by UV–vis absorption spectroscopy. The results of UV–vis spectra indicated that the secondary structure of BSA molecules was affected significantly by the presence of iPA. UV–vis spectra of BSA indicated that on addition of iPA, the absorption peaks in the visible region showed a moderate blue-shift. The overall binding constant K for iPA-BSA complexes is estimated to be 4.9 x 104 M-1 using UV absorption spectroscopy.

Experimental

Chemical Synthesis of iPA.

iPA was prepared following the methods described by Robins.21 Briefly, reaction of adenosine with isopentenyl bromide affords iPA in 40% overall yield. An alternative procedure required more expensive 6-chloropurine riboside and isopentenylamine, but proceeded with 82% yield of the final product.

Biological study

Cell culture

Breast cancer cell lines were maintained in the standard medium and grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 g/ml streptomycin. Cultures were maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

***In vitro* evaluation of cytotoxic activity**

iPA was evaluated for *in vitro* activity against selected human tumor cell lines derived from breast cancer. SRB assay has been used to estimate cell viability or growth.[22,23] iPA stock solutions (10 mM in DMSO) were prepared and stored at 4°C and were diluted with DMEM to 0.1–1 mM range at room temperature before experiment. The final percentage of DMSO in the reaction mixture was less than 1% (v/v). Cancer cells (2×10^3 cells/ well) were plated in 5 multiple in the 96-well plates and incubated in medium for 24 h. Serial dilutions of individual compounds were added. The microtiter plates were incubated at 37°C, 5% CO_2, 95% air, and 100% relative humidity for 72 h prior to addition of iPA. The assay was terminated by the addition of 50 µL of cold trichloroacetic acid (final concentration, 10% TCA) and incubated for 60 min at 4°C.

The plates were washed five times with tap water and air-dried. SRB solution (50 µL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 30 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried or under hood. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm and used as a relative measure of viable cell number. The percentage of growth inhibition was calculated by using the equation: percentage growth inhibition (1-At/Ac) x 100, where At and Ac represent the absorbance in treated and control cultures, respectively. IC_{50} was determined by interpolation from dose–response curves.

Assessment of cell shape and cell morphology

Cell shape was monitored by phase-contrast microscopy. For this morphological study MDA-MB-231 cancer cell line was seeded at a density of 5×10^5 cells/well into 6-well plates. After attachment the cells were incubated in the presence of different concentrations or absence of iPA for different time intervals. Treated and untreated (control) cells were viewed using an inverted phase-contrast microscope model Zeiss HBO 50 and photographed using a Nikon camera attached to the microscope.

Binding study

Purified BSA "essentially globulin and fatty acid free" (Aplichem Co. Germany) was used to prepare the stock 2.5×10^{-5} M solution of by dissolving an appropriate amount of BSA in 0.05 mol L^{-1} Tris-HCl buffer of pH 7.4 containing 0.1 M NaCl solution, then stored at 4°C. The purity of BSA stock solution (10×10^{-3} M in DMSO) was estimated to be 99% based on an absorbance value at 279 nm using a reference value of 0.667 for 1.0 g L^{-1} of pure BSA. All stock solutions were stored at 4 °C in dark. All solutions in this work were diluted to the required volume with Tris–HCl buffer of pH 7.4. All other reagents involved in this work were of analytical-reagent grade, and doubly distilled water was used throughout. UV–vis absorption spectra were measured on a Beckman UV spectrophotometer with the use of a 10 mm quartz cuvette.

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