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Full Length Research Paper

Kinetin (N^6-furfuryladenine): Cytotoxicity against MCF-7 breast cancer cell line and interaction with bovine serum albumin

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N^6-furfuryladenine (kinetin) is a cytokinin growth factor with several biological effects observed in human cells and fruit flies. Kinetin exists naturally in the DNA of almost all organisms tested so far, including human cells and various plants. The cytotoxicity effect of kinetin on MCF-7 breast cancer cell lines was measured by the sulforhodamine B (SRB) assay and the results showed that kinetin could inhibit cell growth in a dose-dependent manner with an IC_{50} value of 52 µmol/L at 96 h after the drug was added into the culture. The interaction of bovine serum albumin (BSA) with kinetin was studied in aqueous solution at physiological conditions, using constant protein concentration and various drug contents. UV-vis spectroscopic method was used to determine kinetin binding mode, the binding constant and the effects of kinetin complexation on protein structure. The spectroscopic results showed that kinetin is located along the polypeptide chains with overall affinity constant of $K_{Kinetin\text{-}BSA} = 6.8 \times 10^4\ M^{-1}$.

Key words: N^6-furfuryladenine (kinetin), MCF-7, cytotoxicity, sulforhodamine B (SRB), bovine serum albumin (BSA).

INTRODUCTION

Cytokinins are purine derivatives with potential anticancer activity, originally discovered as phytohormones that promote cell division, leaf expansion and callus cell redifferentiation. Early and recent experimental evidence suggests that naturally occurring and synthetic cytokinins target human cancer cells through antiproliferative, apoptogenic and differentiation-inducing activities (Ishii et al., 2002; Dolezal et al., 2007; Spinola et al., 2007; Bifulco et al., 2008; Cabello et al., 2009; Laezza et al., 2010; Ottria et al., 2010; Voller et al., 2010; Rajabi et al., 2010). N^6-furfuryladenine (kinetin**,** Figure 1) was first isolated and identified in 1955 (Miller et al., 1955) from DNA as an artifactual rearrangement product of the autoclaving process and as a molecule with multiple biological activities (Barciszewski et al., 2007). Since then this compound has been widely used as a synthetic cytokinin in various aspects of plant research, including applications in biotechnology and cell biology (Rakwal et al., 2003). Most importantly, kinetin also exerts anti ageing effects on plants, as well as on human skin cells and fruitflies (Rattan et al., 1994; Sharma et al., 1995). Nevertheless, It is expected that an anticancer activity may be express by kinetin in which previous studies showed their biological activities and little is known about anticancer activity of kinetin against MCF-7 breast cancer cell line which was derived from a pleural effusion of a patient with metastatic breast cancer and was later recognized as the first hormone-responsive breast cancer cell line (Soule et al., 1973). The usefulness of the MCF-7 cell line as an investigative tool led to its adoption in laboratories worldwide and several decades of use in independent laboratories have facilitated the evolution of distinct MCF-7 lineages (Levenson and Jordan, 1997; Gooch and Yee, 1999).

Serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions. Binding to albumin is an important factor in the solubilization and transportation of drugs in blood and the molecules that have higher affinity

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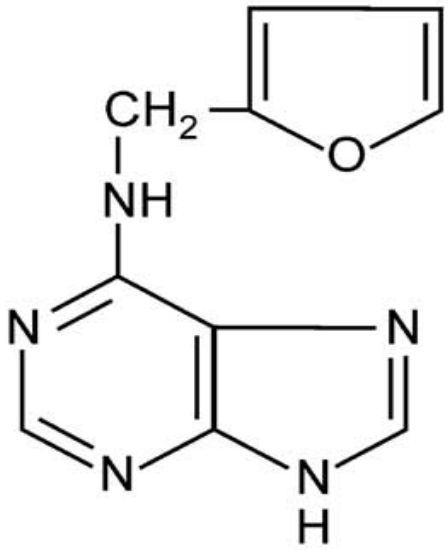

Figure 1. Structure of N^6-furfuryladenine (kinetin).

Figure 2. Structure of bovine serum albumin.

for serum albumin, and show preferential binding sites on serum albumin which may find important therapeutical applications. The most important property of this group of proteins is that they serve as transporters for a variety of compounds. Bovine serum albumin (BSA) (Figure 2) has been one of the most extensively studied of this group of proteins, particularly because of its structural homology with human serum albumin (HSA) (Gelamo et al., 2002; Flarakos et al., 2005; Wang et al., 2007).

In this study, antiproliferative activity of kinetin against MCF-7 breast cancer cell line was studied by sulforhodamine B (SRB) assay. As a complementary information related to the antiproliferative activity, kinetin interaction with BSA has been studied, while analyzing absorbance changes in the UV-vis frequency range with the aim to obtain structural information regarding the kinetin binding mode, apparent binding constant and the effects of conformational changes of native BSA after complexation with kinetin.

MATERIALS AND METHODS

Trypsin, trypan blue, antibiotic and antimycotic agent, fetal bovine serum (FBS), SRB, dimethyl sulfoxide (DMSO) and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals were purchased from Sigma-Aldrich Italia (Milan, Italy).

Cell culture

Human breast cancer MCF-7 cells were supplied from American Type Cell Culture Collection and were maintained in the standard medium and grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37°C with 5% CO_2 in a humidified atmosphere.

In vitro evaluation of cytotoxic activity

Growth activity of kinetin *in vitro* was evaluated by the SRB assay which was used for cell density determination, based on the measurement of cellular protein content. SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mild acidic conditions, SRB binds to the proteins basic amino acid residues in TCA (trichloro acetic acid) fixed cells to provide a sensitive index and cellular protein content that is linear over a cell density range of at least two orders of magnitude (Vichai and Kirtikara, 2006). Kinetin stock solution (10 mM in DMSO) was stored at 4°C and diluted with DMEM up to 0.1 to 1 mM range at room temperature before treatment. The final percentage of DMSO in the reaction mixture was less than 1% (v/v). Cancer cells (2 x 10^3 cells/well) were plated in 96-wells plates and incubated in medium for 24 h. Serial dilutions of individual compounds were added. The plates were incubated at 37°C, for 96 h prior to addition of kinetin. The assay was terminated by the addition of 50 µl of ice-cold trichloroacetic acid (final concentration, 10% TCA) and incubated for 60 min at 4°C.

The plates were washed five times with distilled 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 $(1 - A_t/A_c) \times 100$, where, A_t and A_c represent the absorbance in treated and control cultures, respectively. IC_{50} was determined by interpolation from dose-response curves.

Evaluation of cell morphology

MCF-7 cells, plated at about 20,000 cells/well on chamber slides (8 wells), were treated with 0, 10, 50 and 100 M of kinetin for 96 h. After rinsing in PBS, cells were fixed in methanol and stained with 10% Giemsa and photographed using a Nikon camera attached to the microscope.

Binding study of BSA

Purified BSA "essentially globulin and fatty acid free" (Aplichem Co. Germany) was used to prepare the stock 2.5×10^{-5} M solution 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 the dark. All solutions in this study were diluted to the required volume with Tris–HCl buffer of pH 7.4. All other reagents involved in this study 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.

RESULTS AND DISSCUSION

Cytotoxicity evaluation of kinetin *in vitro*

The human breast cancer cell line MCF-7 provides an unlimited source of homogenous self-replicating material, free of contaminating stromal cells, and can be easily cultured in simple standard media. Such a cell line is ideal to study the interaction between a chemopreventive drug and a cancer cell. The mechanism by which a chemopreventive drug inhibits the proliferation of a cancer cell can be best studied *in vitro* where the other physiological regulatory mechanisms, which are present in the *in vivo* system, are absent. *In vitro* studies provide the advantages, from an experimental point of view, of being able to observe cells and tissues in isolation and away from the controlling and modifying influences of other tissues in the body. In the presence of different doses of kinetin, the cells were inhibited ranging from 10 to 90% with a loss of viable cells (Figure 3). Kinetin inhibited the proliferation of MCF-7 cells in a concentration and time-dependent manner. The viability of the MCF-7 cells was unaffected by less than 5 µmol/L kinetin treatment for 96 h, but changed to 95%, in response to 10 µmol/L kinetin, respectively. However, viability decreased to 50% in response to 52 µmol/L kinetin and continued to decrease as the concentration of kinetin was increased, and was 10% with 100 µmol/L and 5% with 150 µmol/L kinetin exposure. Thus, it was evident that kinetin up to a dose of 20 µmol/L was almost non-cytotoxic *in vitro*. The MCF-7 cells were viable up to 95% at the maximum effective dose of kinetin 10 µmol/L used in the study and viability of control was taken as 100%. The concentration which causes 50% inhibition of cell viability (IC_{50}) by kinetin was 52 µmol/L at 96 h by employing SRB assay (Figure 3B). Further research is required in order to demonstrate that this antiproliferative effect may be related to apoptosis and cell cycle arrest.

Cell morphology and shape study

For more than 150 years, morphological features played the leading role in the description of cell death. However, during the past three decades, cell death has been characterized on the molecular level, which markedly increased our understanding of the morphology 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. Figure 3c shows the incubation of the cells with different concentrations of kinetin after 96 h treatment. Cells in the control group were regular polygonal and cell antennas were short and there were very few round cells. Cells treated with kinetin for 4 days showed obvious morphological changes, including the loss of adhesion, rounding and sporadical distribution, and there was clear concentration-response tendency, indicating the possibility of apoptosis occurrence. Further staining for fluorescence microscopic studies is required to determine whether the cytotoxic effects and morphological alternations of kinetin were related to the induction of apoptosis.

Absorption spectra of kinetin-BSA complexes

The UV spectra of free kinetin and BSA are reported in comparison with kinetin-BSA complex (Figure 4). The increase in intensity of kinetin characteristic UV-vis band at 279 nm is due to major drug-BSA interaction (Nafisi et al., 2008).

The calculation of the overall binding constants was carried out on the basis of UV absorption as reported (Figure 5). The equilibrium for kinetin and BSA complex can be described as follows:

$$[\text{BSA} + \text{kinetin}] \leftrightarrow [\text{BSA - kinetin}]K_{\text{kinetin}}$$

$$K_{\text{kinetin}} = [\text{BSA - kinetin}]/[\text{BSA}][\text{kinetin}]$$

The double reciprocal plot of $1/[A-A_0]$ vs $1/[\text{ligand}]$ is linear and the association binding constant (K) is calculated from the ratio of the intercept on the vertical coordinate axis to the slope. A_0 is the initial absorption of the free BSA at 279 nm and A is the recorded absorption at different kinetin concentrations (L).

The overall binding constant K for kinetin-BSA complexes is estimated to be 6.8×10^4 M^{-1}. The value obtained is indicative of a good kinetin-protein interaction. The reason for the low stability of the kinetin-BSA com-

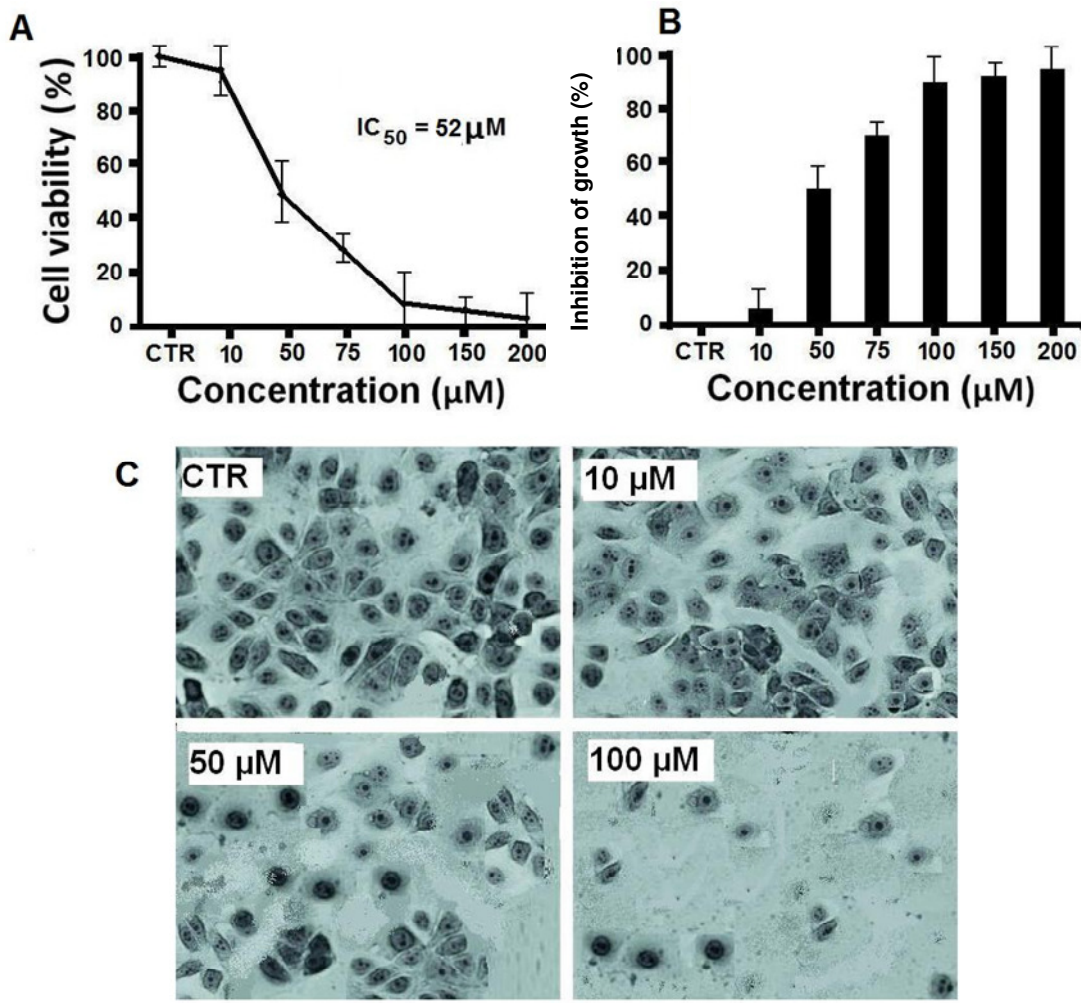

Figure 3. Effects of kinetin on the proliferation of MCF-7. A and B: The percentage of growth inhibition was calculated by using the equation: $(1- A_t/A_c) \times 100$, where A_t and A_c represent the absorbance in treated and control cultures, respectively. IC_{50} was determined by interpolation from dose-response curves; C: morphological analysis of the effects of kinetin on MCF-7 after 96 h incubation.

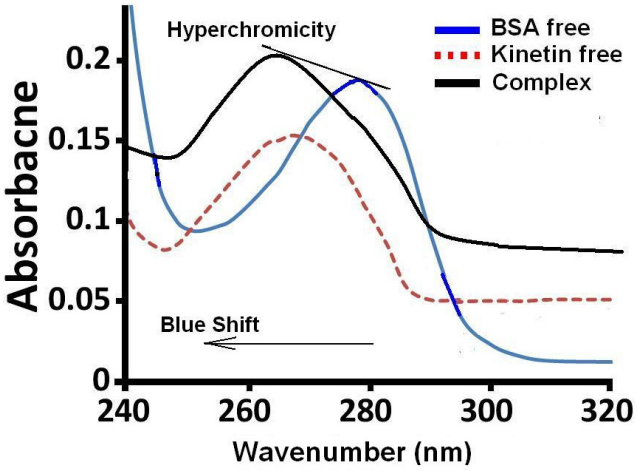

Figure 4. UV-vis absorbance spectra of BSA in the presence of kinetin.

plexes can be attributed to the presence of mainly hydrogen bonding interaction between protein donor atoms and the kinetin polar groups or an indirect drug-protein interaction through water molecules.

Conclusion

The ability to induce cancer cell death is an important property of a candidate anticancer drug. The process of cytotoxicity is characterized by specific biochemical assays and morphological changes. This study demonstrates the antiproliferative properties of kinetin toward MCF-7 human breast cancer cells by the SRB assay and the results showed that kinetin displayed good cytotoxic effect against MCF-7 and could inhibit cell growth in a dose-dependent manner with an IC_{50} value of52 µM at 96 h after the drug was added into the culture.

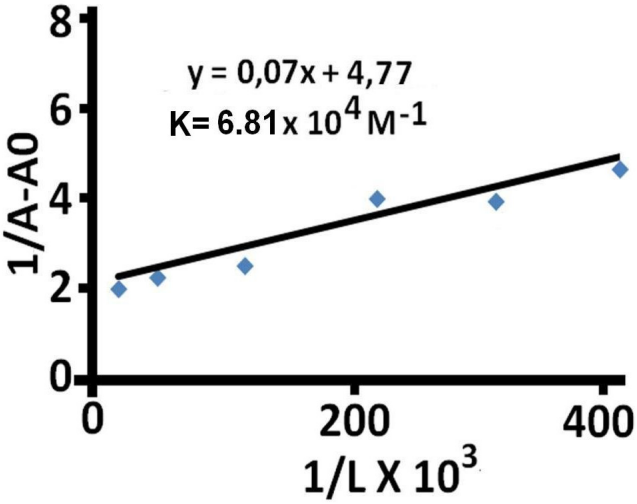

Figure 5. The plot of $1/(A-A_0)$ vs 1/L for BSA and kinetin complexes where A0 is the initial protein absorption band (279 nm) and A is the recorded absorption at different drug concentrations (L).

The cell viability result showed that the MCF-7 cells were sensitive to the toxic higher doses of kinetin than to the low doses. From this results, we strongly suggest the selection of >50 µM concentration of kinetin due to its effect for further studies. The Giemsa staining revealed that there is a morphological change in cells, such as loss of adhesion, rounding, cell shrinkage and detachment from the substratum. Our result from structural analysis showed interaction of kinetin with BSA. Since protein-drug binding greatly influences absorption, distribution, metabolism and excretion properties of typical drugs, studies on the protein-drug binding is important for the elucidation of the reaction mechanisms, providing a pathway to the pharmacokinetics and pharmacodynamic mechanisms of these substances in various tissues.

The interaction of BSA with kinetin was studied in aqueous solution at physiological conditions, using constant protein concentration and various drug contents. UV-vis spectroscopic method was used to determine ligands' binding mode, the binding constant and the effects of ligand complexation on protein secondary structure. UV-vis spectra of BSA indicated that on addition of kinetin, the absorption peaks in the visible region showed a moderate blue-shift. The overall binding constant *K* for kinetin-BSA complexes is estimated to be 6.8 x 10^4 M^{-1} using UV absorption spectroscopy. In conclusion, kinetin may have the potentials for inclusion in an anticancer drug discovery programme based on its role on induction of apoptosis and cell cycle arrest in the near future and further research including morphological staining studies, biochemical assays and flow cytometric analysis are required in order to demonstrate that its antiproliferative effects may be related to apoptosis and cell cycle arrest.

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