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Antiproliferative activity and therapeutic implications of potassium tris(4-methyl-1-pyrazolyl) borohydride in hepatocellular carcinoma

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ABSTRACT

The study of iron chelators as cancer chemotherapeutic agents is still in its infancy. Accordingly, there is a need to optimize new chelating molecules for iron chelation therapy and cancer treatment. Previous studies have demonstrated that the ligand tris(1-pyrazolyl) borohydride and its derivates were able to chelate ferrous iron, but very little research focused on their biological properties and applications in cancer treatment. So, in this study, several boron-pyrazole derivatives were chosen for the examination of their effects on the proliferation of human hepatocellular carcinoma (HCC) cell lines. The results suggested that potassium tris(4-methyl-1-pyrazolyl) borohydride ($KTp^{4\text{-}Me}$) exhibited the most potent anti-tumor activities among the candidates. Hence, the antiproliferative activity and the iron chelating capacity of the iron chelator $KTp^{4\text{-}Me}$ in HCC cell lines HepG2 and Hep3B were characterized. $KTp^{4\text{-}Me}$ could disrupt cell iron uptake and affect signaling pathways of iron regulation in HCC cell lines and induced the expression of TfR1 and HIF-1α in a concentration-dependent manner, which was a typical cell response to iron deficiency. Moreover, $KTp^{4\text{-}Me}$ arrested cell cycle in S phase and induced cell apoptosis in both Hep3B and HepG2 cells. Overall, our results provide a promising starting point and the possibility of the future development and applications of $KTp^{4\text{-}Me}$ in HCC therapy.

1. Introduction

Iron is essential to all cells, and it influences many aspects of cell function such as oxygen transport, catalytic activity, cell cycle regulation and oxidative signaling, etc., [1–3]. Iron plays multiple important roles in cellular processes either directly takes part in prosthetic groups of enzymes or other proteins or indirectly through its contribution to redox reactions [4]. Consequently, the iron levels in mammals should be monitored continuously to avoid iron overload as well as iron deficiency. To accomplish this goal, cellular iron uptake, storage and utilization must be strictly controlled.

In cells, due to its flexible coordination chemistry and redox reactivity, iron catalyses the propagation of reactive oxygen species (ROS) through Fenton reaction [5]. The potent free radical hydroxyl radical (·OH) is known to cause DNA damage, inactivate tumor suppressor genes, and activate oncogenes. These consequences may promote tumorigenicity, thus promoting uncontrolled cell growth [6,7]. Tumor cells have a high demand for iron when compared with their nontransformed counterparts, corresponding to their increased growth rates relative to normal cells [8]. Furthermore, neoplastic cells express higher levels of the iron-containing enzyme, ribonucleotide reductase (RR), which is a critical rate-limiting step for DNA synthesis [9]. Therefore, tumor cells are far more sensitive than normal cells to iron depletion, and iron deprivation is considered as a potential therapy for inhibiting tumor growth.

Hepatocellular carcinoma (HCC) is the third most common reason for cancer-related mortality worldwide [10,11]. Liver is the major site of pathologic iron accumulation, so it is more sensitive

Abbreviations: DFO, deferoxamine; $KTp^{4\text{-}Me}$, potassium tris(4-methyl-1-pyrazolyl)-borohydride; ROS, reactive oxygen species; HCC, hepatocellular carcinoma; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBST, tris buffered saline with Tween 20; BSA, bovine serum albumin; Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; Bp44mT, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone.

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to iron overload than other organs, and the excess iron exposing liver to a higher risk of hepatocellular carcinoma [12]. The current treatment of HCC is based only on cytostatics which have high incidence of side effects. Moreover, treatments for HCC, especially for advanced stages, are very likely to encounter problems of drug resistance [13].

The chelator currently used to treat iron overload disease, desferrioxamine (DFO), has shown anti-proliferative activity against leukemia and neuroblastoma cells *in vitro*, *in vivo* and in clinical trials [14,15]. In a recent study, DFO treatment produced a partial response in two of the patients and stabilized the disease in three patients when conducted on ten patients with chemo-resistant advanced HCC [16]. These findings suggested that iron chelators do suppress human HCC tumor growth and have important implications in the treatment of HCC. However, the efficacy of DFO is severely limited due to its poor ability to permeate cell membranes and the short serum half-life [17]. These limitations have encouraged the development of other iron chelators that are far more effective than DFO.

Previous studies have demonstrated that the ligand tris(1-pyrazolyl) borohydride and its derivates were able to chelate ferrous iron, but very little research focused on their biological properties and applications in cancer treatment. In this study, several boron-pyrazole derivatives were chosen for the examination of their effects on the proliferation of human hepatocellular carcinoma (HCC) cell lines. We showed that $KTp^{4\text{-Me}}$ chelated iron efficiently and afforded potent anticancer effect on human HCC cell lines through iron deprivation and the ensuing inhibition of Fenton-derived hydroxyl radical formation. All the results indicated that iron chelators may have important applications in human HCC therapy and $KTp^{4\text{-Me}}$ could be a promising compound for the treatment of human HCC.

2. Materials and methods

2.1. Cell culture and reagents

Human hepatocellular carcinoma (HCC) cell lines, Hep3B and HepG2, which were all obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C and 5% CO_2. $KTp^{4\text{-Me}}$ and other boron-pyrazole derivatives were synthesized according to the previously reported literature [18]. DMEM and FBS were obtained from Gibco. DFO, ferrozine and ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) were purchased from Sigma–Aldrich, Inc., CellTiter-Blue reagent was provided by Promega.

2.2. Iron chelating efficiency

The iron chelating potency of $KTp^{4\text{-Me}}$ was investigated according to the method of ferrozine assay, wherein the Fe^{2+}-chelating ability of the chelator was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm [19,20]. Briefly, the reaction mixture, containing $KTp^{4\text{-Me}}$ of different concentrations, hydroxylamine hydrochloride (0.05%), $FeSO_4$ (50 μM) and ferrozine (250 μM) was adjusted to a total volume of 200 μl with distilled water, shaken well and incubated for 10 min at room temperature. After incubation, the absorbance spectra from 300 to 700 nm were recorded using Synergy™ 2 multi-detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The absorbance of the mixture was measured at 562 nm against control. The ability of $KTp^{4\text{-Me}}$ to chelate ferrous ion was calculated using the following equation: Chelating effect (%) = $(1 - A_{562sample}/A_{562control}) \times 100$.

2.3. Plasmid DNA degradation assay

Plasmid DNA degradation assays were performed as described [21]. *Escherichia coli* (DH-5α) were transformed with the plasmid pUC19 and grown in LB broth (Luria–Bertani broth, a widely used medium for the growth of bacteria) containing ampicillin. The plasmid DNA was then purified using the plasmid extraction kit (Tiangen, China). Reagents were added to sterile eppendorf tubes in the following order: purified sterile water, $KTp^{4\text{-Me}}$ (concentration as indicated), hydroxylamine hydrochloride (0.05%), $FeSO_4$ (100 μM), hydrogen peroxide (1 mM) and plasmid DNA (200 μg/mL) to a final volume of 20 μl. Samples were incubated at 37 °C for 60 min and all the samples were immediately loaded with 5 μl 5 × loading dye onto a 0.7% agarose gel containing ethidium bromide. Gels were imaged and then densitometrically analyzed using Quantity One software.

2.4. MTT assay

The antiproliferative activity of the candidate compounds was assessed against HepG2 and Hep3B cell lines via the MTT assay as previously described with modification [22]. Briefly, after exposure of cells to compound, culture media was changed by free serum culture media. 20 μl MTT (5 mg/ml in PBS, sigma) were added to each well for 4 h incubation at 37 °C. After this interval, the media were discarded and the resulting formazan crystals were dissolved in DMSO. The optical densities were measured at 570 nm spectral wavelength using microtiter plate reader (Synergy™ 2 Multi-Detection Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). The inhibition rate (%) was calculated as follows: $(1 - A_{570sample}/A_{570control}) \times 100$. The final antiproliferative activity of $KTp^{4\text{-Me}}$ was expressed as IC_{50} values. IC_{50} values were determined from the results of at least three independent tests and calculated from the inhibition curves.

2.5. Detection of intracellular iron concentration

Hep3B cells pretreated with 500 μM $FeSO_4$ for 24 h and then treated with 100 μM $KTp^{4\text{-Me}}$ for another 24 h or the cells were just treated with 100 μM $KTp^{4\text{-Me}}$ alone for 24 h. All the treated cells were suspended in a buffer containing 10 mM Tris buffer, pH 7.2 and 150 mM NH_4Cl and lysed on ice with occasional vortexing. The supernatants were collected by centrifugation at 10,000 rpm for 15 min and the iron concentration were determined using ferrozine assay [23]. The principle of the test is that transferrin-bound iron can dissociate to form ferrous iron at acidic pH and in the presence of suitable reducing agents. These iron ions, which represent the redox active and chelatable forms of iron that are present in the cell cytosol and organelles, can react with ferrozine to produce a magenta-colored complex with absorption maximum at 562 nm. The difference in color intensity at this wavelength between compound treated and untreated cells is proportional to the concentration of iron in cells.

2.6. Western blotting

After treated with $KTp^{4\text{-Me}}$ for 36 h at indicated concentrations, the cultured cells were washed twice with ice cold PBS and lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, supplemented with protease inhibitor cocktail (Sigma) and scraped off the plate. The extract was transferred to a microfuge tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were collected and stored at −80 °C. For Western blot analysis, equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The membranes were then blocked with 5%