Dp44mT Effectively Induces Human Colorectal Carcinoma Cell Apoptosis via mTOR pathway

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Abstract

AIM: To investigate the anticancer mechanisms of di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) in human colon cancer cells. **Background:** Human Colorectal Carcinoma (HCC) is one of the most commonly diagnosed cancers among both males and females. Current research has considered iron chelators as new anti-cancer agents; however, the anticancer activities of iron chelators and their target genes in HCC have not been well investigated. **Methods**: Dp44mT was used to pretreated with two colorectal tumor cell lines SW480 and HT-29. The pro-apoptosis effects of different concentration Dp44mt was measured by both flow-cytometry and Hoechst 33258 staining. Ferric ammonium citrate (FAC) was used as additional iron donor to inhibit the effects of Dp44mT. Apoptosis and DNA damage related proteins was examined by western blot. **Results:** In this study we found that iron chelators Dp44mT could induce the apoptosis in two colorectal tumor cell line SW480 and HT-29, upregulate the expression level of p-Histone H2A.X and inhibit the phosphorylation level of mTor in a dose-dependent way. Those effects could reversed by additional iron donor FAC. **Conclusion**: These data indicate that iron depletion and/or the presence of iron can modulate the HCC apoptosis progression in vitro, which may be an potential target for future HCC therapy.

Key Words: Dp44mT, cell apoptosis, mTOR, DNA damage

Introduction

Colorectal cancer is the fourth most common cause of cancer-related deaths globally, and the annually deaths number has increased to approximately 700, 000[1]. Surgical resection and chemotherapy are the primary treatment. However, the risk of resection surgery and the side effects of chemotherapy including hair loss, neuropathy urging the researchers to find new therapy target for colorectal cancer. Besides, a major limitation of cytotoxi chemotherapy is drug resistance caused by P-glycoprotein (Pgp)[2], which greatly restrict the effects of chemotherapy.

Iron (Fe) is essential for proliferation, and many studies have shown that tumor cells are more sensitive to Fe deprivation than normal cells[3]. This sensitivity probably exists because cancer cells have greater Fe requirements than their normal counterparts and because cancer cells express higher levels of the Fe-containing enzyme, ribonucleotide reductase (RR), which is the critical rate-limiting step in DNA synthesis[4] and this has resulted in the examination of chelators as anti-cancer agents.

As a novel class of anti-tumor agents, the iron chelator, di-2-pyridylketone 4,4-di methyl-3-thiosemicarbazone (Dp44mT) was widely studies in recent years[5]. The ant i-tumor mechanism of Dp44mT was considered to be related with its ability to bi nd, depletion cellular iron and generate cytotoxic radicals[6]. In addition, Dp44mT d emonstrate potent and selective anti-tumor activity[6-8], and could overcome the Pgp-related multidrug resistance by directly utilizing lysosomal Pgp-transport activity[9, 10]. And this agent has entered Phase I clinical trials[11].

Notably, Dp44mT lead to induction of a variety of apoptotic markers, such as cleaved caspase 3, caspase 4, cleaved PARP, etc., in different cancer cell-types[12, 13]. However, the anti-tumor effects of Dp44mT on colorectal tumor cells has not been examined. In this study we investigated the pro-apoptosis effects of Dp44mT on different kinds of colorectal tumor cell lines. Dp44mT induces colorectal tumor cells apoptosis in a dose-dependent way, and the antagonist of Dp44mT, Ferric ammonium citrate (FAC) could reverse those effects. These results indicated that cellular iron might be a possible target for colorectal tumor and Dp44mT might be a potential therapy.

Materials and Methods

Reagents

Dp44mT was synthesized and characterized using standard procedures[14]. Dp44mT was dissolved in DMSO at 10 mM. Stock solutions of ferric ammonium citrate (FAC, Sigma-Aldrich, St Louis, MO, USA) was prepared in deionized water at 100 mg/mL. Both solutions were stored at -20 °C and diluted to the proper concentrations before use. In this experiment, the highest level of DMSO in media was 0.05% (v/v).

Cell culture

The human colon carcinoma cell lines HT29 and SW480 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in 10% FBS-supplemented RPMI medium with L-glutamine and maintained at 37 °C with 5% CO_2.

Flow Cytometry

Flow cytometry was performed as described previously [15]. Briefly, the HT29 and SW480 cells were washed with cold PBS 2 times and then were resuspended in binding buffer (10 mM Hepes/NaOH (pH 7.4), 0.14 M NaCl, and 2.5 mM CaCl2), and FITC Annexin V and PI were added. After incubation at room temperature for 15 min in the dark, flow cytometry was analyzed. Annexin V-FITC and PI double staining were regarded as late apoptotic or necrotic cells.

Hoechst 33258 Staining

Briefly, preparations of fixed cells were rinsed three times with PBS, permeabilised with 70% ethanol (30 s), and incubated with a solution of Hoechst 33258 (2 µg/mL) for 30 min at room temperature. Then, the cells were observed by fluorescence microscope (Leica, Germany).

Protein Extraction and Western Blotting

Cells were harvested and washed with cold phosphate-buffered saline solution. Cells were suspended in lysis buffer and the lysate was then sonicated, centrifuged (16,000 x g/40 min/4 °C), and the supernatant collected for further analysis. The proteins were quantitated by the standard Bradford assay. Equal amounts of protein from the treated cells were loaded and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and then transfered onto nitrocellulose membranes. The blotted membrane was incubated with antibodies against p-Histone H2A.X, mTOR, p-mTOR and GAPDH, followed by incubation with a secondary antibody conjugated to horseradish peroxidase. All antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). The chemiluminescence signals were captured using a ChemiDoc MP Imaging System (BioRad; NSW, Australia). Densitometric analysis was performed using ChemiDoc Image Lab Software (BioRad).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of 3 experiments. Experimental data were compared using one-way ANOVA. Results were considered statistically significant when $p<0.05$.

Results

Dp44mT induces SW480 and HT-29 cells apoptosis

The Annexin V-FITC/PI double staining and Hoechst 33258 staining were applied to evaluate the effect of Dp44mT on SW480 and HT-29 cells apoptosis. The results of flow cytometry demonstrated that more apoptotic cells were countered in Dp44mT-treated groups compared with 0 μM group (Figure 1A and 1B). The apoptotic percentage in the different concentration of Dp44mT-treated group were significantly higher than that in the 0 μM treated group ($p<0.05$, Figure 1C and 1D). Besides, the effects of Dp44mT on SW480 and HT-29 cells apoptosis is dose dependent. Treated by 10 μM Dp44mT showed the strongest effect on SW480 and HT-29 cells apoptosis. Therefore, 10 μM Dp44mT was chosen for the followed experiments.

After Hoechst 33258 staining, the SW480 and HT-29 cells in the 0 μM Dp44mT group showed normal shape with round intact nuclei (Figure 2), whereas the Dp44mT-treated cells became more scarce and showed reduced nuclear size, extensive blebbing, strong fluorescent spot, and pyknotic nuclei. These indicate condensed chromatin and apoptotic bodies. Consistent with the results of flow cytometry, Dp44mT induced SW480 and HT-29 cells apoptosis in a dose-dependent way.

FAC inhibited Dp44mT-induced SW480 and HT-29 cells apoptosis

Flow cytometry was performed to assess the rescued effects of FAC on Dp44mT-induced SW480 and HT-29 cells apoptosis. In this experiment, 10 μM Dp44mT was chosen to induce the cell apoptosis, percentages of apoptotic cell in untreated control group, Dp44mT group, FAC group (100 mg/ml) and Dp44mT + FAC group were compared (Figure 3). The results showed that co-treated with FAC could significantly inhibited the cell apoptosis induced by Dp44mT. Similar results were observed in both SW480 and HT-29 cells.

Dp44mT induced SW480 and HT-29 cells apoptosis via mTOR pathway

Effects of different concentration of Dp44mT and FAC on protein expressions of p-Histone H2A.X, mTOR and p-mTOR in SW480 cells was investigated. As shown in Figure 4, Dp44mT induced protein expressions of p-Histone H2A.X and inhibited phosphorylation of mTOR in a dose dependent way. Cells treated with Dp44mT and FAC showed lower expression of p-Histone H2A.X and higher expression of mTOR and p-mTOR compared with the Dp44mT treated cells. These results indicated that FAC inhibited Dp44mT-induced p-Histone H2A.X expression and recovered phosphorylation of mTOR in SW480 cells.

Discussion

Increased drug resistance to standard treatment among cancers lead to the investig ation of new therapeutic strategies. As a result, the implication of the Dp44mT an d its analogues was emerging as new anti-cancer therapeutics, suggested by their broad anti-tumor activity [6, 16, 17] and their effects on drug resistance [18] and tumor metastasis [19], oral bioavailability as well as tolerability. Di-2-pyridylketone 4-cyclo hexyl-4-methyl-3-thiosemicarbazone, kind of Dp44mT analogues, entered multi-cent er clinical trials for the treatment of advanced and resistant tumors in early 2016 [11].

In the present study, effects of different concentration of Dp44mT on colorectal cancer cells apoptosis were explored. The results indicated that Dp44mT could induce cell apoptosis in a dose-dependent way. FAC, which was usually used as the agonist of Dp44mT, could significantly inhibited cell apoptosis induced by Dp44mT. The mechanisms exploration indicated that the pro-apoptotic effects of Dp44mT on colorectal cancer cells were related to promoted expression of p-Histone H2A.X and inhibition of mTOR and p-mTOR. The rescued effects of FAC partially contributed to its inhibition on Dp44mT-induced p-Histone H2A.X expression and recovery of phosphorylation of mTOR.

Previous studies have shown that the ability of a chelator to bind cellular Fe leads to apoptosis[20]. If Fe chelators lead to tumor cell death by influence the apoptotic pathway, then regulating apoptosis becomes important for inhibiting cancer cell proliferation.

Because Dp44mT was the most effective chelator yet screened for antitumor activity, it was crucial to assess its ability to induce apoptosis.

In this study, we found that Dp44mT could promote the expression of p-Histone H2A.X, which indicating the double-strand DNA damage. The PI3K/AKT/mTOR pathway plays a critical role in the growth and progression of colorectal cancer. As the downstream protein of PI3K/Akt pathway, mTOR play an import role in the cell proliferation and apoptosis. In our study we investigated the influence of Dp44mT on the phosphorylation level of mTOR, and found that Dp44mT could inhibit its activation significantly. This may partly explain the anti-tumor effects of De44mT and related iron chelator.

In conclusion, in this study, we investigated the anti-tumor effects of iron chelators Dp44mT on colorectal tumor cells. We found that Dp44mT could promote the tumor cell apoptosis and upregulate the expression level of p-Histone H2A.X and inhibit the phosphorylation level of mTOR in a dose-dependent way. These data indicate that iron depletion could modulate the HCC apoptosis progression in vitro, which may be an potential target for future HCC therapy.

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