Inhibition of Colon Cancer Cell Growth by Imidazole Through Activation of Apoptotic Pathway

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Background: This study aimed to investigate the inhibitory effect of imidazole on colon cancer cell proliferation and understand the mechanism involved.

Material/Methods: MTT assay and flow cytometry using Hoechst 33258 staining were used to assess cell proliferation and morphology, respectively. Changes in protein expression was determined by western blotting assay. The reactive oxygen species (ROS) production in DLD-1 cells was analyzed by flow cytometry using DCFH-DA (2’,7’-dichlorofluorescein diacetate) stain.

Results: DLD-1 and HCT-116 cell viability was suppressed by imidazole in a concentration-based manner. At the concentration of 36 μM, imidazole reduced DLD-1 and HCT-116 cell viability to 22% and 28%, respectively. Treatment with imidazole led to chromatin material condensation, detaching of cells, and apoptotic nuclei. In imidazole treated cells, the G1/G0 phase cell proportion increased, whereas in the S and G2/M phases the cell proportion decreased. Imidazole treatment of DLD-1 cells markedly promoted activation of caspase-3, caspase-8, and caspase-9. The level of cleaved PARP1 was also upregulated in DLD-1 cells with imidazole treatment. Treatment of DLD-1 cells with imidazole suppressed Bcl-2 and promoted Bax, p53, and cytc expression. The Akt activation was suppressed by imidazole treatment in DLD-1 cells. ROS generation in DLD-1 cells was enhanced markedly by treatment with imidazole.

Conclusions: The present study demonstrated that imidazole inhibited colon cancer cell viability through activation of apoptosis and cell cycle arrest by increasing the generation of ROS, caspase activation, and apoptotic protein expression. Therefore, imidazole can act as a therapeutic molecule for the treatment of colon cancer.

MeSH Keywords: Antioxidants • Apoptosis • Chemoembolization, Therapeutic • Chromatin Assembly and Disassembly

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Background

Colorectal cancer, a commonly detected carcinoma, accounts for more than 10% of the deaths caused by cancer throughout the world [1]. The most common site of metastasis for colorectal cancer is the liver, and it is reported that even at a primary stage, colorectal cancer metastases to liver in around 15% of patients [2]. Moreover, in more than 50% of patients, colorectal cancer ultimately metastasizes to the liver during the course of progression [2]. The characteristic feature of colorectal cancer is that surgical extraction from metastasis sites like the liver and lungs increases the survival rate of patients [3]. The chemotherapy agents available so far do not increase the survival rate of patients with colorectal cancer [4]. Development of effective treatment either as monotherapy or combination chemotherapy is urgently required for the colon cancer patients. Most of the anti-cancer molecules activate apoptosis, which is a regular process for getting rid of undesired cells from the body during development or homeostasis [5,6]. The characteristic changes in apoptotic cells include shrinkage, condensation of the chromatin, altered mitochondrial membrane potential, and genetic material fragmentation [5,6]. In addition, the extrinsic pathway for apoptosis caspases are activated by the receptors in membranes, and in the intrinsic pathway, different stress stimuli activate apoptosis [7,8]. Generally, tumor cells are eliminated from the body by synthetic therapeutic agents and natural products that take the advantage of apoptotic pathways [9,10].

Heterocyclic molecules have been frequently used as scaffolds by medicinal chemists in drug discovery programs [11]. Several complex biologically potent natural products and therapeutic molecules have been found to contain imidazole as the main structural component [12]. The compounds containing imidazole exhibit a wide spectrum of biological activities including anti-microbial [13], anti-tumor [14], anti-Alzheimer [15], anti-HIV (human immunodeficiency virus) [16], anti-malarial [17] properties. Two potent candidates against Mycobacterium tuberculosis pretomanid and delamanid, also contain 4-nitroimidazole as their structural component [18]. Delamanid was approved by the Food and Drug Administration (FDA) for the treatment of patients infected with MDR-TB and pretomanid is currently under clinical trials for the treatment tuberculosis patients [19]. Taking into account these biological activities of imidazole bearing compounds, the present study was designed to investigate the effect of imidazole on colon cancer cell viability. The study demonstrated that imidazole inhibits proliferation of colon cancer cells by activation of cell apoptosis.

Material and Methods

Cell line and culture conditions

DLD-1 and HCT-116 colon carcinoma cells were provided by the Chinese Academy of Sciences (Shanghai, China). The cell culture was performed in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. In addition, penicillin (100 U/mL) and streptomycin (100 U/mL) were also mixed with the medium. The conditions used to culture the cells in an incubator were humidified atmosphere of 5% CO₂ at temperature of 37°C.

MTT assay

DLD-1 and HCT-116 cells were put into 96-well microtiter plates at 3×10³/mL concentration in DMEM. Following culture for 12 hours, fresh medium mixed with 0.5, 1.0, 1.5, 3, 6, 12, 24, and 36 μM concentration of imidazole was added to the plates and incubation was carried out for 48 hours. MTT solution (10 μL, having a concentration of 0.5 mg/mL) was then added to the plates and cell incubation was continued for 4 hours. The crystalline formazan formed in the plates was dissolved by adding 80 μL of DMSO followed by absorbance measurement at 573 nm. The measurements were performed 3 times to determine the average values.

Morphological examination of the cells

In DLD-1 cell cultures, alterations in morphology following imidazole exposure for 48 hours were assessed using Hoechst 33258 staining. Cells were exposed to imidazole at 12, 24, and 36 μM concentrations for 48 hours and then washed with phosphate-buffered saline (PBS) twice for 10 minutes. The cells were then subjected to fixing for 15 minutes at 4°C in 4% formaldehyde solution. Subsequently, staining of the cells was performed for 15 minutes with 0.5 μg/mL solution of Hoechst 33258 stain at room temperature. Morphological alterations in DLD-1 cells were examined by fluorescence microscope (Nikon Eclipse Ti-s, Nikon Corp., Tokyo, Japan).

Cell cycle analysis

Briefly, DLD-1 cells at 1.5×10⁴ cells/mL concentration were put into the 6-well plates and exposed for 48 hours to imidazole at 12, 24, and 36 μM concentrations. Then the cells collected were re-suspended in PBS (300 μL) at room temperature for 45 minutes under complete darkness. The PBS also contained propidium iodide (PI) (0.03 mg) and RNase (60 μg). The DNA content distribution was examined by flow cytometry on Quanta SC (Beckman Coulter, Fullerton, CA, USA).
Analysis of apoptosis

DLD-1 cell apoptosis on exposure to imidazole was examined by Annexin V-FITC/PI assay. The cells were exposed for 48 hours to imidazole at 12, 24, and 36 μM concentrations at 2×10^6 cells/mL density. Following 48-hour exposure, the cells were subjected to PBS washing 2 times for 15 minutes and subsequently put into 250 μL of binding buffer. Incubation of the cells was performed with Annexin V-FITC (5 μL) and PI (5 μL) under darkness at room temperature for 20 minutes. Flow cytometry (Quanta SC, Beckman Coulter) was used to determine the apoptotic cell percentage.

Western blot analysis

DLD-1 cells at 1×10^7 cells/mL density were exposed for 48 hours to imidazole at 12, 24, and 36 μM concentrations. The harvested cells were lysed with lysis buffer [40 mM tris-hydrochloric acid (pH 7.6), ethylenediamine tetraacetate (10 mM), sodium chloride (120 mM), dithiothreitol (1 mM), and Nonide P-40 (0.1%)]. The protein samples were resolved on 10% to 12% sodium dodecyl sulfate (SDS) polyacrylamide gel by loading 30 μg/lane samples. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes which were previously treated with 3% non-fat milk in TBST (tris-buffered saline and Tween-20) for 1.5 hours at room temperature to block the non-specific sites. The membrane incubation with primary antibodies was performed at 4°C overnight. The antibodies were used against Bax, Bcl-2, Akt, p-Akt, p53, cyt-c, caspase-3, caspase-8, caspase-9 and PARP1. The membrane washing with TBST was followed by incubation for 2 hours with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies at room temperature. The blots formed were visualized using the ECL (enhanced chemiluminescence) detection kit (Thermo, USA).

ROS generation analysis

The generation of reactive oxygen species (ROS) in DLD-1 cells by imidazole was analyzed by flow cytometry using DCFH-DA (2’,7’-dichlorofluorescein diacetate) stain. Briefly, the cells were put at 2×10^6 cells/well concentration in 6-well plates and exposed to 12, 24, and 36 μM concentrations of imidazole for 48 hours. Then cells were harvested, washed with PBS and subsequently put into a 10 μM solution of DCFH-DA in PBS and incubated for 35 minutes at 37°C. The PBS washed cells were examined using flow cytometry for ROS production.

Statistical analysis

The values are presented as average ± standard deviation (SD) for triplicate experiments carried out independently. Determination of statistical differences were performed using one-way analysis of variance (ANOVA) and Student’s t-test.

Results

Effect of imidazole on cell viability

Imidazole was added to DLD-1 and HCT-116 cell cultures at 0.5, 1.0, 1.5, 3, 6, 12, 24, and 36 μM concentrations and cell viability was assessed at 48 hours (Figure 1). DLD-1 and HCT-116 cell viability was suppressed by imidazole in a concentration-based manner. At 0.5, 1.0, 1.5, 3, 6, 12, 24, and 36 μM concentrations of imidazole, the viability of DLD-1 cells was suppressed to 95%, 91%, 85%, 73%, 61%, 53%, 34%, and 22%, respectively. HCT-116 cell viability was decreased to 96%, 93%, 88%, 76%, 64%, 55%, 43%, and 28%, respectively on treatment with 0.5, 1.0, 1.5, 3, 6, 12, 24, and 36 μM concentrations of imidazole.

Imidazole altered DLD-1 cell morphological features

DLD-1 cells following 48 hours of treatment with imidazole were stained with Hoechst 33258 for morphological examination (Figure 2). Treatment with imidazole at 12, 24, and 36 μM concentrations led to chromatin material condensation, detaching of cells and apoptotic nuclei. However, the untreated cells were normal in shape with no evident chromatin material condensation.

Effect of imidazole on DLD-1 cell cycle

DLD-1 cell cycle progression was analyzed following treatment with 12, 24, and 36 μM concentrations of imidazole by
FACS (Figure 3). In imidazole treated cells in the G1/G0 phase, cell proportion increased significantly with the enhancement in concentration. Treatment with imidazole reduced DLD-1 cell proportion in the S and G2/M phases significantly in comparison to the untreated cell cultures. In the G1/G0 phase, imidazole treatment at 12, 24, and 36 μM concentrations increased cell proportion to 43.78%, 49.23%, and 57.65%, respectively. The cell proportion in the S phase decreased to 17.32%, 12.76%, and 9.18%, respectively on treatment with 12, 24, and 36 μM concentrations of imidazole.

Effect of imidazole on apoptosis

In DLD-1 cells treatment with 12, 24, and 36 μM concentrations of imidazole was followed by flow cytometry of the Annexin/PI stained cells (Figure 4). Imidazole treatment of DLD-1 cell cultures raised the apoptotic cell count significantly in comparison to the untreated cultures. At 12, 24, and 36 μM concentrations of imidazole the count of apoptotic cells reached 17.86%, 29.21%, and 7.98%, respectively. In control DLD-1 cell cultures, the proportion of apoptotic cells was only 1.98%.

Imidazole promoted activation of caspases

Imidazole treatment of DLD-1 cells markedly promoted activation of caspase-3, caspase-8, and caspase-9 in a concentration-based manner (Figure 5). A marked increase in caspase-3, caspase-8, and caspase-9 in DLD-1 cells was caused by 12, 24, and 36 μM concentrations of imidazole. The level of cleaved PARP1 was also upregulated in DLD-1 cells on treatment with imidazole.
Imidazole promoted pro-apoptotic protein expression in DLD-1 cells.

Treatment of DLD-1 cells with 12, 24, and 36 μM concentrations of imidazole suppressed Bcl-2 and promoted Bax expression (Figure 6). The levels of p53 and cyt-c were increased in DLD-1 cells on treatment with imidazole at 12, 24, and 36 μM concentrations. The activation of Akt was suppressed by imidazole treatment in DLD-1 cells at 48 hours.

Imidazole generated ROS in DLD-1 cells

ROS generation in DLD-1 cells by imidazole at 12, 24, and 36 μM concentrations was measured using DCFH-DA staining (Figure 7). Treatment with imidazole markedly upregulated production of ROS in DLD-1 cells in concentration-based manner. The ROS production in DLD-1 cells was maximum on treatment with 36 μM concentrations of imidazole at 48 hours.
Discussion

Heterocyclic compounds containing the imidazole ring have been shown to exhibit diverse biological activities like anti-tumor [14], anti-HIV [16], anti-malarial [17], activity. The present study was designed to demonstrate the effect of imidazole on colon cancer cell growth in vitro. The study demonstrated that imidazole inhibited DLD-1 and HCT-116 cell viability through the apoptotic pathway and production of ROS. Most of the drugs used to treat carcinomas are associated with the activation of apoptosis through mitochondrial pathways [20–22]. Alteration of mitochondrial membrane potential is believed to be an important indication of apoptosis onset [23–26]. Changed membrane potential initiates efflux of cytochrome c which consequently activates caspase-3 and caspase-9 [23–26]. In the present study, imidazole exposure exhibited inhibitory effect on DLD-1 and HCT-116 cell proliferation in a concentration-based manner. The apoptotic features were clearly visible in DLD-1 cells on exposure to imidazole for 48 hours. Exposure of DLD-1 cells to imidazole caused a marked increase in the proportion of apoptotic cells in comparison to control cultures. Apoptosis activation by anti-cancer compounds has been mainly explored to treat various types of cancers [5,6]. The apoptotic cells have characteristic features which differentiate them from normal cells [5,6]. The extrinsic pathway for apoptosis is associated with activation of caspases by the receptors in membranes, while the intrinsic pathway is initiated by different stress stimuli [7,8]. The Bcl-2 protein is contained in the membranes of the nucleus, endoplasmic reticulum, and mitochondria where it plays a role as radical scavenging agent by quenching the radicals produced [27,28]. Increased expression of Bax in the mitochondria is associated with the onset

![Figure 6. Effect of imidazole on pro-apoptotic proteins in DLD-1 cells. Western blotting was employed to analyze changes in Bcl-2, Bax, p53, cyt-c, and Akt levels in DLD-1 cells by imidazole treatment.](image)

![Figure 7. Effect of imidazole on reactive oxygen species (ROS) production in DLD-1 cells. Production of ROS in DLD-1 cells following treatment with different concentrations of imidazole was measured by flow cytometry of DCFH-DA stained cells. Magnification 200×.](image)
of apoptotic cell death [29]. The ratio of Bax/Bcl-2 expression must be higher in the cells undergoing apoptosis [30,31]. The results from our current study showed that imidazole exposure in DLD-1 cells promoted the level of Bax and suppressed the level of Bcl-2 expression. The ratio of Bax/Bcl-2 was enhanced significantly by imidazole treatment in DLD-1 cells. The ROS production has also been found to act as signaling molecule for activation of cell apoptosis [32–35]. Results from our current study revealed generation of ROS at a higher level on exposure of DLD-1 cells to different concentrations of imidazole. It has been reported that Akt on phosphorylation acts as an anti-apoptotic molecule and prevents cells from undergoing apoptosis [36]. Phosphorylated Akt downregulates activity of caspase-8 by promoting the expression of FLICE inhibitory protein [37]. In our current study, exposure of DLD-1 cells to imidazole suppressed the phosphorylation of Akt. These findings proved that imidazole activated apoptosis in DLD-1 cells by downregulation of Akt activation.

Conclusions

The present study demonstrated that imidazole inhibited colon cancer cell viability through activation of apoptosis and cell cycle arrest by increasing the generation of ROS, caspase, and apoptotic protein expression. Therefore, imidazole can act as a therapeutic molecule for the treatment of colon cancer.

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