

Small molecule 2,3-DCPE induces S phase arrest by activating the ATM/ATR-Chk1-Cdc25A signaling pathway in DLD-1 colon cancer cells

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Abstract. In our previous study, it was reported that 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol (2,3-DCPE) induces apoptosis and cell cycle arrest. The current study aimed to investigate the molecular mechanism involved in 2,3-DCPE-induced S phase arrest. The results demonstrated that 2,3-DCPE upregulated phosphorylated (p-)H2A histone family member X, a biomarker of DNA damage, in the DLD-1 colon cancer cell line. Western blotting revealed that 2,3-DCPE increased the checkpoint kinase (Chk1) (Ser317 and Ser345) level and decreased the expression of M-phase inducer phosphatase 1 (Cdc25A) in a time-dependent manner. Subsequently, the results demonstrated that the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) inhibitors wortmannin and caffeine had no effect on the cell cycle; however, the inhibitors partially abrogated 2,3-DCPE-induced S phase arrest. Flow cytometry assays revealed that caffeine (2 mM) reduced the proportion of S phase cells from 83 to 39.6% and that wortmannin (500 nM) reduced the proportion of S phase cells from 83 to 48.2%. Furthermore, wortmannin and caffeine inhibited the 2,3-DCPE-mediated phosphorylation of Chk1 and the degradation of Cdc25A. However, these ATM/ATR inhibitors had limited effect on 2,3-DCPE-induced apoptosis. Taken together, the data of the current study indicated that 2,3-DCPE caused DNA damage in colon cancer cells and that

2,3-DCPE-induced S phase arrest was associated with the activation of the ATM/ATR-Chk1-Cdc25A pathway.

Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-associated mortality in the United States, according to the statistics update in 2020 (1). Systemic therapies, including 5-Fluorouracil (Fu)-based chemotherapy, molecular-targeted therapy and immunotherapy, have improved the 5-year relative survival rate of patients with CRC to 65%, according to statistics in 2019 (2). However, non-specific cytotoxic antitumor agents usually induce side effects and decrease patient tolerance to treatment (3). Furthermore, drug resistance remains a challenge, leading to the failure of CRC treatment (4). In addition to gaining an understanding of the mechanism of intrinsic and acquired therapy resistance, researchers have focused on small-molecule compounds that induce less toxicity and have greater efficacy for cancer treatment (5,6). In our previous study, a chemical library obtained from ChemBridge Corporation was screened for potential novel anticancer agents. The results demonstrated that a synthetic compound, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol (2,3-DCPE), inhibits cell proliferation and induces apoptosis and cell cycle arrest in CRC cells (7).

The cell cycle is a process comprised of complex and consecutive changes involved in cell proliferation (8). Cell cycle arrest, one of the DNA damage responses (DDR) to DNA repair and apoptosis, is determined according to severity of DNA damage (9). In response to DNA damage or DNA replication blockage, cell cycle progression can be stalled in the G₁, S or G₂ phase (9). This cell cycle arrest mechanism serves as a protective system by which cells can repair damage and maintain genomic stability (9). Ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR), members of phosphatidylinositol 3-kinase-related kinase family of proteins, are two important DDR transducers that interact with p53, checkpoint kinase (Chk)1, Chk2 and CDK (9). Certain investigators have reported S phase

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arrest in cancer cells treated with various chemotherapeutic agents, including 5-Fu, mitomycin and cisplatin (10,11). The ATM/ATR pathway is involved in S phase arrest through the activation of Chk1 or Chk2 (12).

Our previous study demonstrated that 2,3-DCPE induced S phase arrest, which was also mediated by activation of the p53-independent ERK pathway in DLD-1 human colon cancer cells (7). Additionally, 2,3-DCPE-induced S phase arrest may be blocked by the ATM inhibitors wortmannin and caffeine (7). These observations indicated that, in addition to the function of the ERK pathway, other mechanisms were involved in 2,3-DCPE-induced S phase arrest, which prompted the investigation of the current study. The present study aimed to investigate the molecular mechanism that is associated with 2,3-DCPE-induced S phase arrest by *in vitro* experiments.

Materials and methods

Cells and cell culture. The DLD-1 human colon cancer cell line was obtained from the American Type Culture Collection. The cells were maintained in RPMI-1640 supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 1% glutamine and 1% antibiotics, and cultured at 37°C in a humidified incubator containing 5% CO₂.

Chemicals. DMSO was purchased from Sigma-Aldrich; Merck KGaA and 2,3-DCPE was purchased from ChemBridge Corporation. 2,3-DCPE was dissolved in DMSO at 20 mM to create a stock solution. Wortmannin and caffeine were obtained from Sigma-Aldrich; Merck KGaA.

Drug treatment. Exponentially proliferating DLD-1 cells were continuously exposed to 2,3-DCPE. The cells were treated with 20 μM 2,3-DCPE for 8, 10, 12, 14, 16, 18, 24 and 32 h to investigate the effect of cell cycle arrest and DDR-associated proteins. DMSO alone was used as the control because it does not have any effect on cells. Cells were pretreated in wortmannin (500 nM) or caffeine (2 mM) for 2 h and 2,3-DCPE (20 μM) was added and incubated for another 24 h to investigate the effect of ATM/ATR inhibition on S phase arrest. Cells cultured in DMSO were used as controls. Cells were cultured under different pretreatment conditions (ATM/ATR inhibitors or controls), as aforementioned, for 2 h and then treated with 2,3-DCPE (20 μM) for a further 32 h to detect the effect of ATM/ATR inhibitors on 2,3-DCPE-induced apoptosis. Each experiment was performed, at least, in triplicate.

Flow cytometry assays. Following treatment, suspended DLD-1 cells were collected separately and adherent cells were trypsinized. Then, the cells were pooled and centrifuged at 2,000 × g at 4°C for 5 min prior to being fixed in 70% ethanol overnight at 4°C. Following this, the cells were stained with propidium iodide for analysis of DNA content. Flow cytometry was performed at the Flow Cytometry Core Laboratory at our institution as described previously (7).

Western blotting. DLD-1 cells were rinsed with ice-cold PBS and lysed in Laemmli lysis buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl). Protein concentration was determined using

the BCA Assay kit (cat. no. 23227, Pierce; Thermo Fisher Scientific). Equal amounts (20 μg/lane) of total cellular proteins were loaded onto a 10% polyacrylamide gel, resolved using SDS-PAGE and subsequently transferred to a PVDF membrane (Amersham; Cytiva). The membrane was blocked for 1 h at room temperature in phosphate-buffered saline containing 0.05% Tween-20 (PBST) supplemented with 5% non-fat dry milk. The membrane was incubated overnight at 4°C with the following primary antibodies: Phosphorylated (p)-Chk1(Ser317 and Ser345) and p-Chk2 (Ser19, Ser33/35, Thr68) (Cell Signaling Technology, Inc., cat. nos. 12302, 2348, 2666, 2665 and 2661, respectively; 1:1,000 dilution), mouse anti-human Chk1/2 (Santa Cruz Biotechnology, Inc., cat. no. sc-8408/sc-17747, 1:1,000 dilution), mouse anti-human Cdc25A (Santa Cruz Biotechnology, Inc., cat. no. sc-7389, 1:1,000 dilution) and mouse monoclonal anti-phosphorylated-H2A histone family member X (p-H2A.X; Ser 139; Upstate Biotechnology, Inc., cat. no. 613401, 1:1,000 dilution). β-actin (Cell Signaling Technology, Inc., cat. no. 4970, 1:1,000 dilution) was used as the loading control. Following three washes with PBST, the membrane was incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-rabbit/mouse IgG; Cell Signaling Technology, Inc., cat. no. 7074/7076, 1:2,000 dilution). After three washes with PBST, immunoreactivity was observed using an ECL kit (Amersham; Cytiva).

Statistical analysis. Statistical analysis was performed using SPSS statistical software (version 21.0; IBM Corp.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. One-way ANOVA and Bonferroni's correction post hoc analysis were used for comparison between multiple groups. The histograms were plotted using Graph Pad Prism software (version 6.0; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

DNA damage is induced by 2,3-DCPE. To investigate the potential molecular mechanism of 2,3-DCPE as an anticancer treatment, DLD-1 colon cancer cells were treated with 20 μM 2,3-DCPE for different durations (8, 10, 12, 14, 16 and 18 h) as a single agent. The cells were harvested and changes in the cell cycle following treatment were analyzed using flow. 2,3-DCPE induced an increase in the proportion of cells in the S phase in a time-dependent manner (Fig. 1A). Additionally, the presence of DNA damage was evaluated by measuring H2A.X levels. As shown in Fig. 1B, p-H2A.X levels were markedly increased in the cells treated with 20 μM 2,3-DCPE for 14, 16 and 18 h compared with the DMSO-treated group, while the expression of total H2A.X did not exhibit a marked change. Since H2A.X is phosphorylated in the initial stage of DNA double-strand breaks (DSBs) (9), these findings indicated that 2,3-DCPE may induce DSBs accompanied by cell cycle arrest in the S phase.

2,3-DCPE-induced S phase arrest is associated with the activation of Chk1 and the degradation of Cdc25A. Subsequently, the expression levels of DDR-related proteins, including Chk1 and Chk2, were evaluated by western blotting. The results

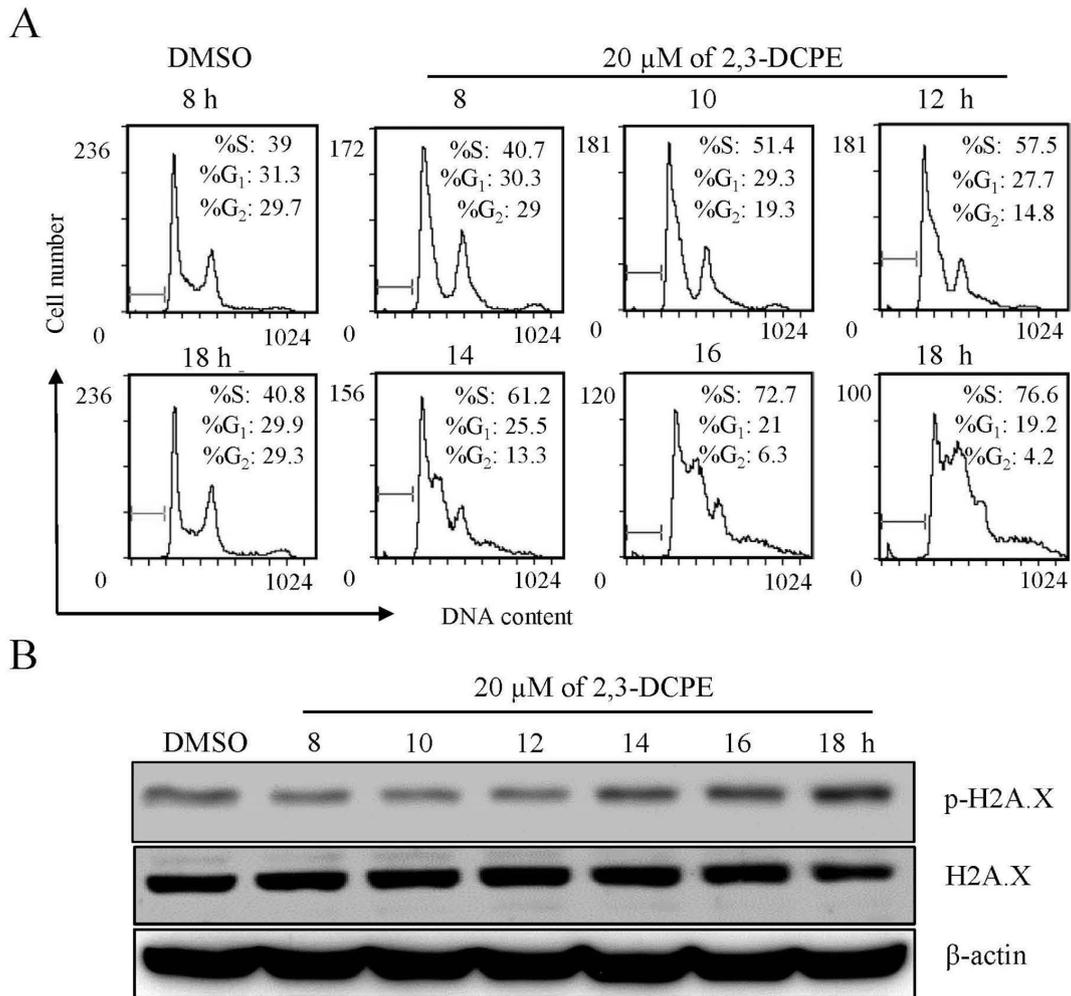


Figure 1. Effect of 2,3-DCPE on S phase arrest and p-H2A.X expression in the DLD-1 cell line. Cells were treated with 20 μM 2,3-DCPE for 8, 10, 12, 14, 16 or 18 h. (A) Cell cycle distribution is presented for each experimental condition. (B) p-H2A.X and total H2A.X levels in the cellular extracts were determined by western blotting with specific antibodies. β-actin was used as an internal control. 2,3-DCPE, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol; p-, phosphorylated; H2A.X, H2A histone family member X.

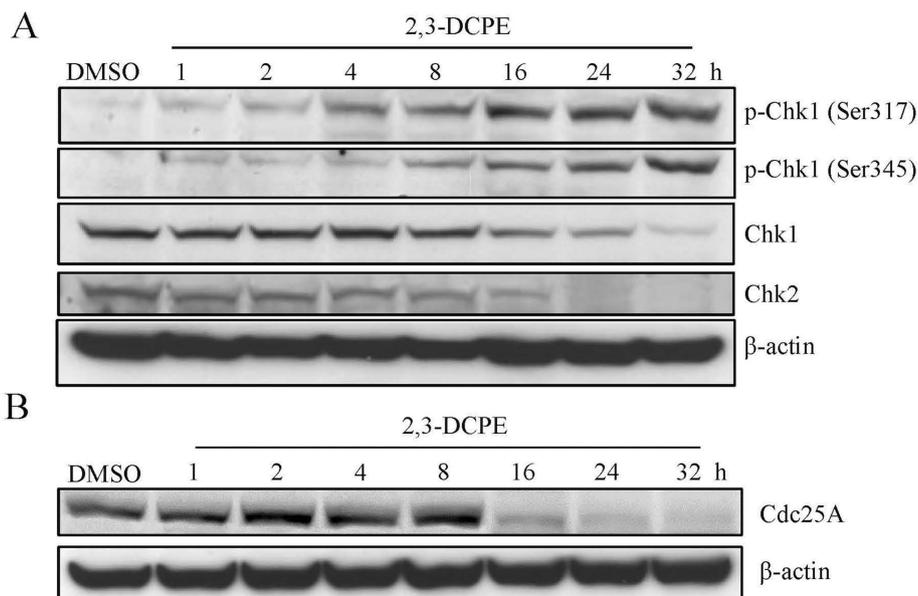


Figure 2. Expression of DNA damage response-related proteins following 2,3-DCPE treatment. (A) Expression level of p-Chk1 (Ser317 and Ser345) was markedly increased and (B) Cdc25A expression was markedly decreased following treatment with 20 μM 2,3-DCPE for 16, 24 and 32 h. 2,3-DCPE, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol; p-, phosphorylated; Chk, checkpoint kinase; Cdc25A, M-phase inducer phosphatase 1.

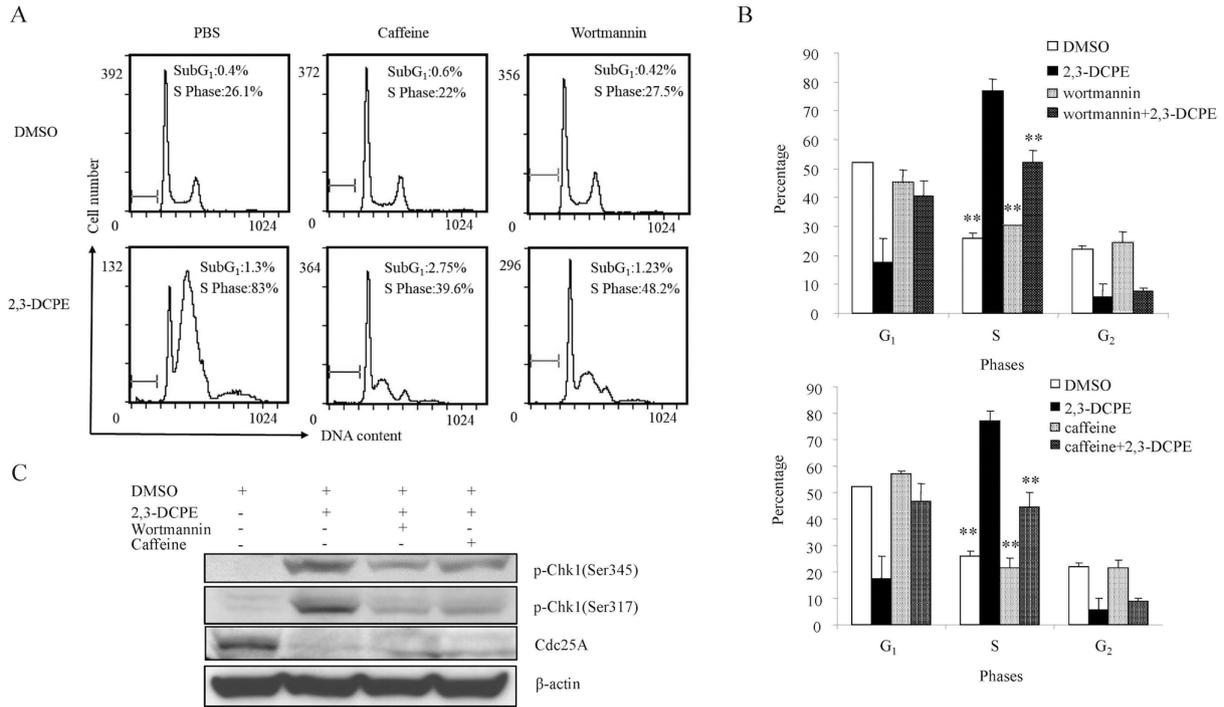


Figure 3. Effect of ATM inhibitors on the 2,3-DCPE-induced cell cycle arrest in the S phase. (A) Cell cycle distribution was determined by flow cytometry assays and (B) the quantitative analysis is presented in the bar chart. Data are expressed as the mean ± standard deviation. **P<0.01 vs. the 2,3-DCPE group. (C) Western blotting was performed to investigate the p-Chk1 and Cdc25A expression after cells were treated with 2,3-DCPE and wortmannin or caffeine. 2,3-DCPE, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol; p-, phosphorylated; Chk, checkpoint kinase; ATM, ataxia-telangiectasia mutated; Cdc25A, M-phase inducer phosphatase 1; DMSO, dimethyl sulfoxide.

indicated that the expression level of p-Chk1 (Ser317 and Ser345) was markedly increased; however, the total levels of Chk1 and Chk2 were markedly decreased in the cells treated with 20 μM 2,3-DCPE for 16, 24 and 32 h compared with the DMSO-treated group (Fig. 2A). There was no difference between the different phosphorylation sites of Chk2 following treatment with 20 μM 2,3-DCPE, including at sites Ser19, Ser33/35, and Thr68 (data not shown). Cdc25A phosphatase is one of the key targets of the checkpoint machinery that ensure genetic stability (13). The most important mechanism of Cdc25A function in regulating cell cycle progression is the dephosphorylation of cyclin D-dependent kinases (CDK4 and CDK6), which leads to the transition into S phase (13). Therefore, the effects of 2,3-DCPE on the expression of Cdc25A in the DLD-1 cells were examined. The down-regulation of Cdc25A resulting from 2,3-DCPE treatment is presented in Fig. 2B. The results demonstrated that 2,3-DCPE decreased the expression of Cdc25A in a time-dependent manner. Therefore, the data indicated that DDR induced by 2,3-DCPE may involve the Chk1-Cdc25A signaling pathway.

ATM/ATR inhibition blocks 2,3-DCPE-induced S phase arrest. As an elementary investigation of the molecular mechanism of 2,3-DCPE-induced S phase arrest, experiments with ATM/ATR pathway inhibitors (wortmannin and caffeine) on the DLD-1 cell line were performed. The results demonstrated that neither wortmannin nor caffeine as a single agent affected the cell cycle; however, when cells were treated with wortmannin/caffeine + 2,3-DCPE, the inhibitors partially blocked 2,3-DCPE-induced S phase

arrest (Fig. 3A and B). Caffeine reduced the proportion of S phase cells from 83% in the 2,3-DCPE/PBS-treated group to 39.6% in the caffeine/2,3-DCPE group, and wortmannin reduced the proportion of S phase cells from 83 to 48.2% in the wortmannin /2,3-DCPE group. Moreover, wortmannin and caffeine markedly inhibited the phosphorylation of Chk1 (Ser345 and S317) and subsequently suppressed the degradation of Cdc25A (Fig. 3C). These findings indicated that the ATM-Chk1-Cdc25A pathway may be critical for 2,3-DCPE-induced S phase arrest in DLD-1 colon cancer cells.

ATM/ATR inhibitors have a limited effect on apoptosis. To further verify the apoptotic effects of ATM/ATR inhibitors on DLD-1 cells, apoptosis rates were analyzed using flow cytometry. As presented by Fig. 4, caffeine increased the percentage of cells in sub-G₁ from 8.56 to 11.9%; however, this difference was not notably different. The apoptotic sub-G₁ peak was not notably different in cells treated with wortmannin. These data indicated that, under these conditions, ATM/ATR inhibitors do not affect 2,3-DCPE-induced apoptosis.

Discussion

Our previous study demonstrated that 2,3-DCPE induced S phase arrest and p21 overexpression; these results was also observed in ATM-defective cancer cells (7). These findings indicated that the antitumor effect of 2,3-DCPE may not depend on ATM. However, molecular mechanisms underlying ATM and the corresponding signaling pathway in cell cycle arrest remain to be elucidated. The present study verified that

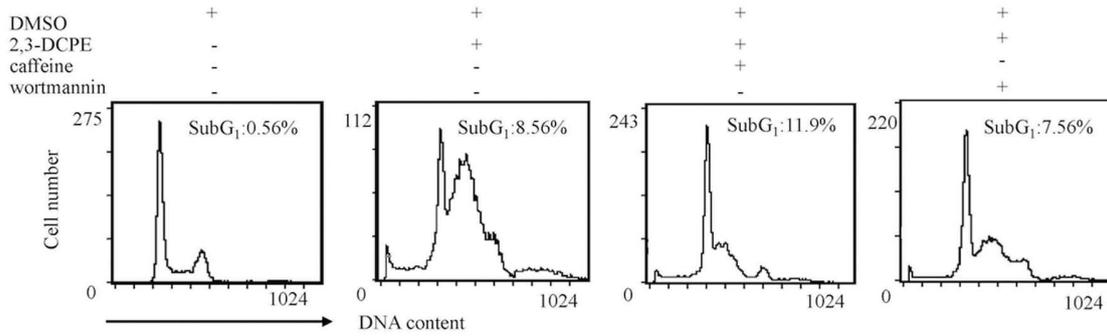


Figure 4. Effect of wortmannin and caffeine on apoptosis. Flow cytometry assays were performed to determine the proportion of cell undergoing apoptosis following different treatment conditions. 2,3-DCPE, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol. DMSO, dimethyl sulfoxide.

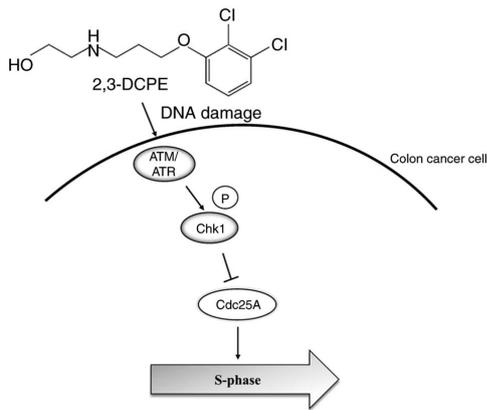


Figure 5. The working model of 2,3-DCPE in colon cancer cells. 2,3-DCPE induced DNA damage and activated ATM/ATR. S phase arrest was induced by the subsequent phosphorylation of Chk1 and the degradation of Cdc25A. 2,3-DCPE, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol; Chk, checkpoint kinase; ATM, ataxia-telangiectasia mutated; ART, ataxia-telangiectasia and Rad3-related; Cdc25A, M-phase inducer phosphatase 1.

2,3-DCPE induced DNA damage and S phase arrest via the ATM-Chk1-Cdc25A pathway (Fig. 5).

Common therapies for colon cancer, including chemotherapy and radiotherapy, effectively kill cancer cells by inducing DNA damage, the most deleterious type of which being the DNA DSB (14). The phosphorylation of H2A.X is considered an initial event in DSBs that leads to the subsequent DDR (15). Certain agents utilized for cancer therapy, including traditional chemotherapeutic drugs and small-molecule compounds, increase H2A.X levels. The increase in H2A.X is associated with the susceptibility of cancer cells to treatment options (16-18). In the present study, 2,3-DCPE increased p-H2A.X levels in DLD-1 cells in a time-dependent manner, indicating its DNA-damaging effect.

To maintain the normal process of the cell cycle, the DDR system is activated to modulate and repair DNA damage, in which the cell cycle checkpoint is the key regulatory mechanism (8). The G₁/S checkpoint is controlled by Chk2, while the G₂/M checkpoint is regulated by Chk1; thereafter, checkpoint kinases regulate cyclins or cell division cycle genes to induce corresponding cell cycle arrest (19). Numerous agents used for CRC therapy regulate checkpoints and affect the cell cycle (20). For instance, cisplatin activates the G₂/M checkpoint and decelerates the replication phase, whereas

oxaliplatin regulates the G₁/S checkpoint and blocks the G₂/M transition (21). Our previous study demonstrated that 2,3-DCPE-induced S phase arrest may be mediated by the activation of the p53-independent ERK/p21 pathway in DLD-1 human colon cancer cells (7). The data of the current study demonstrated that p-Chk1 (Ser317 and Ser345) was upregulated and Cdc25A was downregulated in the DLD-1 cells treated with 2,3-DCPE in a time-dependent manner. However, the expression of p-Chk2 was not significantly altered. It has been reported that Chk1 serves an important role in cell proliferation, cell cycle and apoptosis in colon cancer (22,23). Numerous therapeutic agents act on Chk1. For example, lidamycin, an enediyne antibiotic, acts as an antitumor agent on colon tumor cells by increasing the phosphorylation of Chk1 and Cdc25C, and the expression of cyclin B, causing cell arrest in the G₂ phase (24). Loratadine damages cell DNA in colon cancer cells, thereby activating Chk1 and promoting arrest in G₂/M (25). Similar to the results of the current study, it was reported that 5-Fu induces S phase arrest by activating Chk1. Furthermore, Chk1 downregulation abrogates this arrest and sensitizes colon cancer cells to 5-Fu treatment (26). The inhibition of Chk1 inducing chemosensitivity will be a novel focus for future studies.

Moreover, the data of the current study demonstrated that ATM/ATR inhibitors, wortmannin and caffeine, partially blocked 2,3-DCPE-induced S phase arrest and inhibited the phosphorylation of Chk1 and the degradation of Cdc25A. The role of the ATM/ATR signaling pathway in DDR has been extensively investigated. This pathway is activated when intracellular DNA is damaged and leads to cell cycle arrest in the S phase by the subsequent phosphorylation of Chk1 and the degradation of Cdc25A (19,27). Additionally, a previous study reported that ATM inhibition induces chemoresistance to 5-Fu therapy (9). The data from the current study indicated that the ATM/ATR-Chk1-Cdc25A pathway was involved in 2,3-DCPE-induced S phase arrest in the DLD-1 colon cancer cells.

The western blotting results in our previous study and flow cytometry analysis in the current study demonstrated 2,3-DCPE-induced apoptosis in colon cancer cells (28). Notably, in the present study, when cells are further treated with ATM/ATR inhibitors, 2,3-DCPE-induced S phase arrest is partially blocked without a notable effect on apoptosis. It was hypothesized that cell cycle arrest is a protective response

to DNA damage. Abrogation of the S phase checkpoint causes excessive accumulation of DNA damage and induces apoptosis (29). In the present study, caffeine and wortmannin had limited effects on 2,3-DCPE-induced apoptosis. These results may be partially explained by the fact that caffeine and wortmannin are non-specific inhibitors with low potency and by the intricate mechanisms of 2,3-DCPE-induced cell cycle arrest and apoptosis; however, these outcomes require further exploration.

There were certain limitations in the current study. Other proteins related to ATM/ATR pathway, including cyclin B and cdk2, were not detected. Whether ATM/ATR is the direct target of 2,3-DCPE requires more detailed elaboration by western blotting and mass spectrometry.

In conclusion, the results of the current study demonstrated that 2,3-DCPE induced S phase arrest via activation of the ATM/ATR-Chk1-Cdc25A pathway in DLD-1 colon cancer cells, furthering our understanding of 2,3-DCPE in colon cancer therapy.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BB, LS, JW, JH, WZ, YL, KC, DX and HZ contributed to the conception and design of the current study. Material preparation, data collection was performed by BB, LS and JW. Data analysis and the first draft of the manuscript was written by JH, WZ and YL. Writing review and editing were performed by KC, DX and HZ. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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