Dipyridamole induces the phosphorylation of CREB to promote cancer cell proliferation

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Abstract. Dipyridamole, a traditional anti-platelet drug, has been reported to inhibit the proliferation of cancer cells. The present study aimed to investigate the possibility of dipyridamole as an adjuvant of chemotherapy by enhancing the cytotoxicity of an anti-cancer drug. The cytotoxicity of colorectal cancer cells (HCT-8), $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells and lymphoma cells (U937) to dipyridamole and/or doxorubicin was evaluated using MTT proliferation and colony forming assays. The expression levels of phosphorylated cAMP-regulatory element-binding protein (pCREB) and poly(ADP-ribose) polymerase-1 (PARP-1) in cells were analyzed via western blotting and immunofluorescence. The present study reported controversial data regarding the anti-cancer effect of dipyridamole. Dipyridamole increased, rather than inhibited, the proliferation of HCT-8 and U937 cells in a dose-dependent manner. Furthermore, it was found that dipyridamole significantly increased the expression levels of pCREB and PARP-1. However, the combined usage of dipyridamole significantly enhanced the cytotoxicity of doxorubicin to HCT-8 cells at particular doses. Based on the current findings, dipyridamole likely induces the phosphorylation of CREB to promote the proliferation of cancer cells, but may enhance the cytotoxicity of anti-cancer drugs at particular doses.

Introduction

Dipyridamole, a traditional anti-platelet agent, is an inhibitor of phosphodiesterase enzyme 3 (PDE3) and PDE5, which results in the accumulation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (1), consequently increasing protein kinase A (PKA) and PKG, respectively (2). Previous studies have reported that dipyridamole enhances the cytotoxicity of anti-tumor drugs, such as 5-flurouracil (3), cisplatin (4) and methotrexate (5,6), as well as preventing tumor progression (7) and decreasing the proliferative activity of cancer cells (2,8). Although dipyridamole has been reported to inhibit the re-uptake of adenosine and downregulate cyclin D1 and c-Myc levels (7), the precise role and the relevant mechanism of dipyridamole in regulating the proliferation of cancer cells is yet to be elucidated. Moreover, there is no consensus on the anti-cancer effect of dipyridamole.

Complex signaling pathways are known to mediate the proliferation, survival and therapeutic resistance of cancer cells (9). Of which, cAMP-regulatory element-binding protein (CREB) has been demonstrated to serve a critical role in both hematologic and non-hematologic malignancies (10,11) via the phosphorylation of various kinases, such as Akt and PKA (12,13). Moreover, the cAMP/PKA axis has been demonstrated to regulate poly(ADP-ribose) polymerase-1 (PARP-1) (14), which is known to serve an important role not only in DNA repair (15), but also the development and progression of malignant tumors (16,17). As dipyridamole may induce the accumulation of cAMP and cGMP to support the survival and proliferation of cells, the precise role of dipyridamole on the proliferation of cancer cells requires further investigation.

Doxorubicin is a widely prescribed chemotherapeutic drug, but its use in high doses is limited due to serious side effects, such as myelotoxicity and cardiotoxicity (18). Considering the favorable safety profile of dipyridamole, it will be beneficial to use dipyridamole as adjuvant drug for enhancing the sensitivity of cancer cells to doxorubicin. Therefore, the present study aimed to investigate the anti-cancer effect of dipyridamole in combination with doxorubicin.

Using human colorectal cancer cells (HCT-8), $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells and human monocyte histiocytic lymphoma cells (U937), it was identified that dipyridamole increased, rather than inhibited, the proliferation of HCT-8 and U937 cells in a dose-dependent manner. However, the cytotoxicity of doxorubicin was

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enhanced by the combined usage of dipyridamole at particular doses.

Materials and methods

Cell culture. Human colorectal cancer cells (HCT-8), $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells and human monocyte histiocytic lymphoma cells (U937) were used for the experiments. Cells were maintained in RPMI-1640 medium (Fujifilm Wako, Inc.) supplemented with 10% FBS (Corning, Inc.) and 1% penicillin/streptomycin (Fujifilm Wako, Inc.), at 37˚C in a humidified atmosphere with 5% CO_2 and 95% air.

Cytotoxicity assay. Based on previous publications (2,7,8), we used 0, 10 and 20 μM dipyridamole in this study. For doxorubicin, we used the same dose of 0, 1.0 and 3.0 μM for HCT-8 cells as our previous study (19). However, the cytotoxicity of doxorubicin largely vary among cancer cell lines (20). As cytotoxicity of doxorubicin to U937 cells was highly indicated even at dose of 0.5 μM in our preliminary experiment, we used 0, 0.1 and 0.2 μM doxorubicin for U937 cells in this study. Cytotoxicity assay was performed using the Cell Proliferation Kit I (MTT) according to the manufacturer's protocol (Roche Diagnostics). Briefly, cells were seeded in 96-well culture plates ($5x10^3$ cells/well) and cultured overnight. Cells were then treated with various concentrations of doxorubicin (Fujifilm Wako, Inc.) and dipyridamole (Sigma-Aldrich; Merck KGaA). At 24 h after treatment, MTT was added and incubated for another 4 h. The formation of formazan from MTT was stopped by adding solubilization solution, and the absorbance of formazan was measured at 570 nm using a microplate reader (iMark™ Microplate Reader; Bio-Rad Laboratories, Inc.). The optical density (OD) value of cells with vehicle treatment was used as a normalization control (100%). The combination effect was analyzed with coefficient of drug interaction [CDI = AB/(A x B)], where AB is the OD value ratio of the combination group and vehicle groups, A is the OD value ratio of the drug A and vehicle groups, and B is the OD value ratio of the drug B and vehicle groups. CDI value <1 indicates a synergistic effect, CDI value =1 indicates an additive effect and CDI value >1 indicates an antagonistic effect. Optical density (OD); coefficient of drug interaction (CDI).

Colony forming assay (CFA). A CFA was performed to confirm the MTT assay data of dipyridamole in regulating the proliferation of parent and $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells. After treatment with 20 μM dipyridamole for 24 h, the cells were collected, re-cultured in 6-well culture plate (200 cells/well) and incubated for 10 days. Colonies were fixed with 4% formalin (Fujifilm Wako, Inc.) for 20 min and stained with 0.5% crystal violet solution for 1 h. Colonies were counted using ImageJ 2.1.0 software (National Institutes of Health).

Western blotting. Western blotting was performed as previously described (21). Briefly, cells were lysed in Laemmli's buffer. Total proteins were separated using SDS-PAGE and were then transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). After blocking, the membranes were incubated with primary antibodies against rabbit phosphorylated (p)CREB (Ser133; 1:1,000, cat. no. ab32096; Abcam), rabbit PARP-1 (1:1,000 cat. no. 9542; Cell Signaling Technology, Inc.) and mouse α-tubulin (1:1,000, cat. no. 3873; Cell Signaling Technology, Inc.) which was followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies against rabbit (1:2,000, cat. no. p0448; Dako Agilent Technologies) and mouse (1:2,000, cat. no. p0260; Dako Agilent Technologies). The expression was visualized using an ECL detection kit (cat. no. RPN2106; Cytiva). Images were acquired using ImageQuant LAS 4000 Mini biomolecular imager (Cytiva). Semi-quantification on the relative expression of proteins was performed using ImageJ 2.1.0 software (National Institutes of Health).

Immunofluorescence staining. Immunofluorescence staining was performed to detect the expression of pCREB. Briefly, cells were fixed with 4% formalin (Fujifilm Wako, Inc.) for 10 min. After blocking, cells were incubated with primary antibodies against pCREB (1:100) at room temperature for 1 h, followed by incubation with an Alexa Fluorescent 546-conjugated secondary antibody against rabbit Ig (1:500 cat. no. A11035; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h in the dark. The cell nuclei were labeled with DAPI.

Statistical analysis. Data are presented as the mean ± SEM for three independent experiments. The data were analyzed using one-way ANOVA, followed by Tukey's multiple comparison post-test. $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed using GraphPad Prism 8.0 software or Excel Microsoft 365.

Results

Dipyridamole increases the proliferation of cancer cells. A MTT assay is used to evaluate the proliferation of cancer cells. The addition of dipyridamole (0-20 μM) increased the proliferation of U937 cells, parent HCT-8 cells and the $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells in a dose-dependent manner (Fig. 1A).

To further assess the data of the MTT assay, a colony forming assay was conducted for parent and $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells. Dipyridamole (20 μM) increased the number of colonies for parent HCT-8 cells (from 748±70 to 866±172; P=0.33) and $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells (from 761±122 to 1106±176; P=0.04) (Fig. 1B). Based on these findings, dipyridamole may promote, but not inhibit the proliferation of cancer cells.

Dipyridamole enhances the expression levels of pCREB and PARP-1. Western blotting was performed to investigate the potential mechanism on the proliferation of cancer cells induced by dipyridamole. It was identified that the expression of pCREB in all cancer cells was increased at 30 or 60 min after 20 μM dipyridamole treatment (Fig. 2A). The enhanced expression of pCREB was also confirmed via immunofluorescence staining (Fig. 2B). Furthermore, the expression of PARP-1 in all cancer cells was enhanced at 30 or 60 min after 20 μM dipyridamole treatment (Fig. 3).

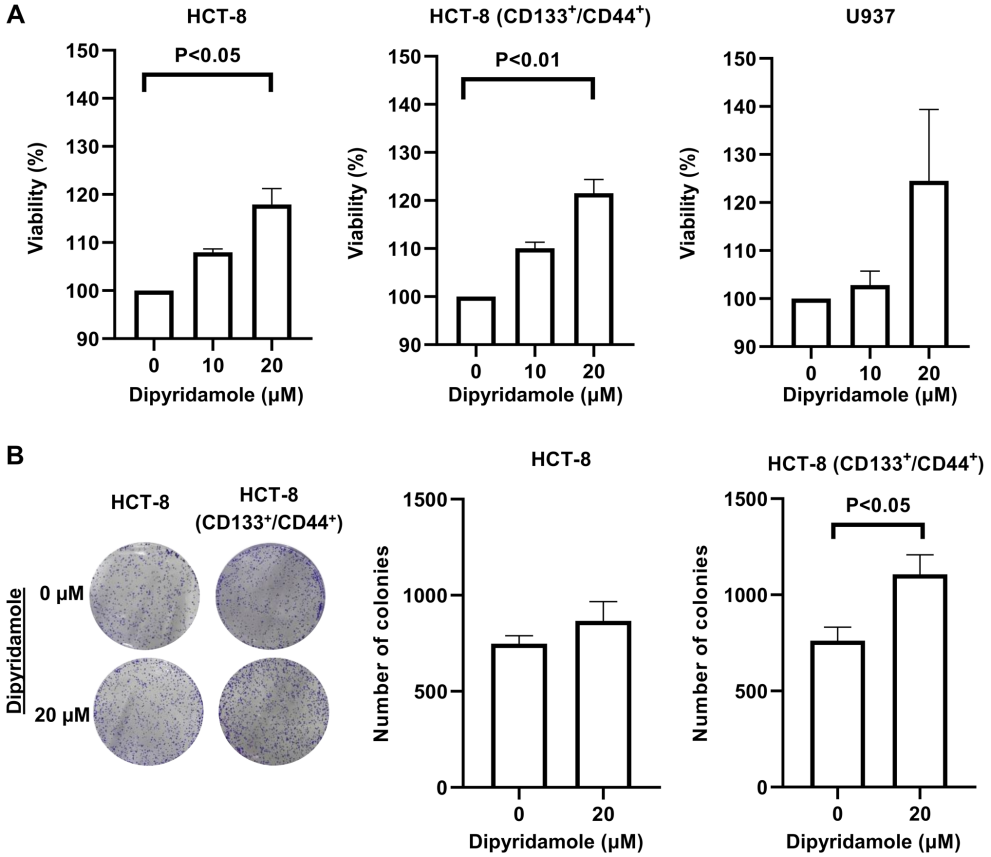

Figure 1. Proliferation of HCT-8 cells, CD133+/CD44+ stem-like subpopulation of HCT-8 cells and U937 cells. (A) MTT assay was used to assess the viability of cancer cells treated with various doses of dipyridamole. (B) Colony forming assay was used to assess the proliferation of parent and CD133+/CD44+ stem-like subpopulation of HCT-8 cells.

Cytotoxicity of doxorubicin is enhanced by dipyridamole at particular doses. To evaluate whether dipyridamole enhances the cytotoxicity of doxorubicin to cancer cells, various doses of dipyridamole were added to cancer cells in combination with doxorubicin treatment. The correct doses for both drugs were chosen according to the reported literature and preliminary experiments (19,20), and the IC_{50} values of doxorubicin were calculated as 5.95 μM for HCT-8 cells, 10.63 μM for the subpopulation of CD133+/CD44+ HCT-8 cells and 0.29 μM for U937 cells (Fig. S1). As indicated by the quantitative data of the MTT assay (Fig. 4; Table SI), dipyridamole significantly enhanced the sensitivity of cancer cells to doxorubicin, but only at a particular dose (10 μM dipyridamole with 3 μM doxorubicin for HCT-8 cells). However, dipyridamole at any of the other doses failed to demonstrate a significant enhancement of doxorubicin cytotoxicity to these cancer cells (Fig. 4; Tables SII and SIII).

Discussion

Dipyridamole, one of the most commonly used anti-platelet agents, is also often prescribed to some patients with cancer. Previous studies have reported that dipyridamole sensitizes cancer cells to chemotherapeutic agents (2-7). Although dipyridamole exerts an anti-proliferative effect on breast and prostate cancer cells (2,7), the anti-cancer benefit of dipyridamole to patients with cancer is yet to be fully elucidated.

The present study aimed to investigate the precise role on the anti-cancer benefit of dipyridamole. In contrast to previous studies (2-7), the present results suggested that dipyridamole increased the proliferation of parent HCT-8 cells, CD133+/CD44+ stem-like subpopulation of HCT-8 cells and U937 cells in a dose-dependent manner. Although the current study neither evaluated cAMP or cGMP levels, nor the activation of cAMP/PKA or cGMP/PKG signaling pathways, the increased cell proliferation induced by dipyridamole could be explained as follows: PDE3 and PDE5 are extensively expressed in healthy tissue cells (22), and upregulated in multiple cancer cells, including HCT-8 and U937 (13,23). As an inhibitor of PDE3 and PDE5, dipyridamole usually induces the accumulation of cAMP and cGMP in cells (1). cAMP and cGMP are generally known to activate PKA and PKG, respectively, which induces the phosphorylation of CREB (13,24) and pCREB regulates the expression of several genes involved in the metabolism, proliferation, differentiation and survival of cells (12). Thus, we speculate that the increased cellular levels of cAMP and cGMP contribute to the small beneficial effect of dipyridamole on the cell survival/proliferation (up to around 20% by MTT assay). Moreover, the present results indicated that dipyridamole increased the expression of pCREB in U937 lymphoma cells, parent HCT-8 colorectal cancer cells and the CD133+/CD44+ stem-like subpopulation from HCT-8 cells. Although we have not yet investigated, it is possible that other PDE3 and PDE5 inhibitors may also beneficial of cell proliferation. The current findings suggested that dipyridamole enhanced the expression of PARP-1, which known to support the survival

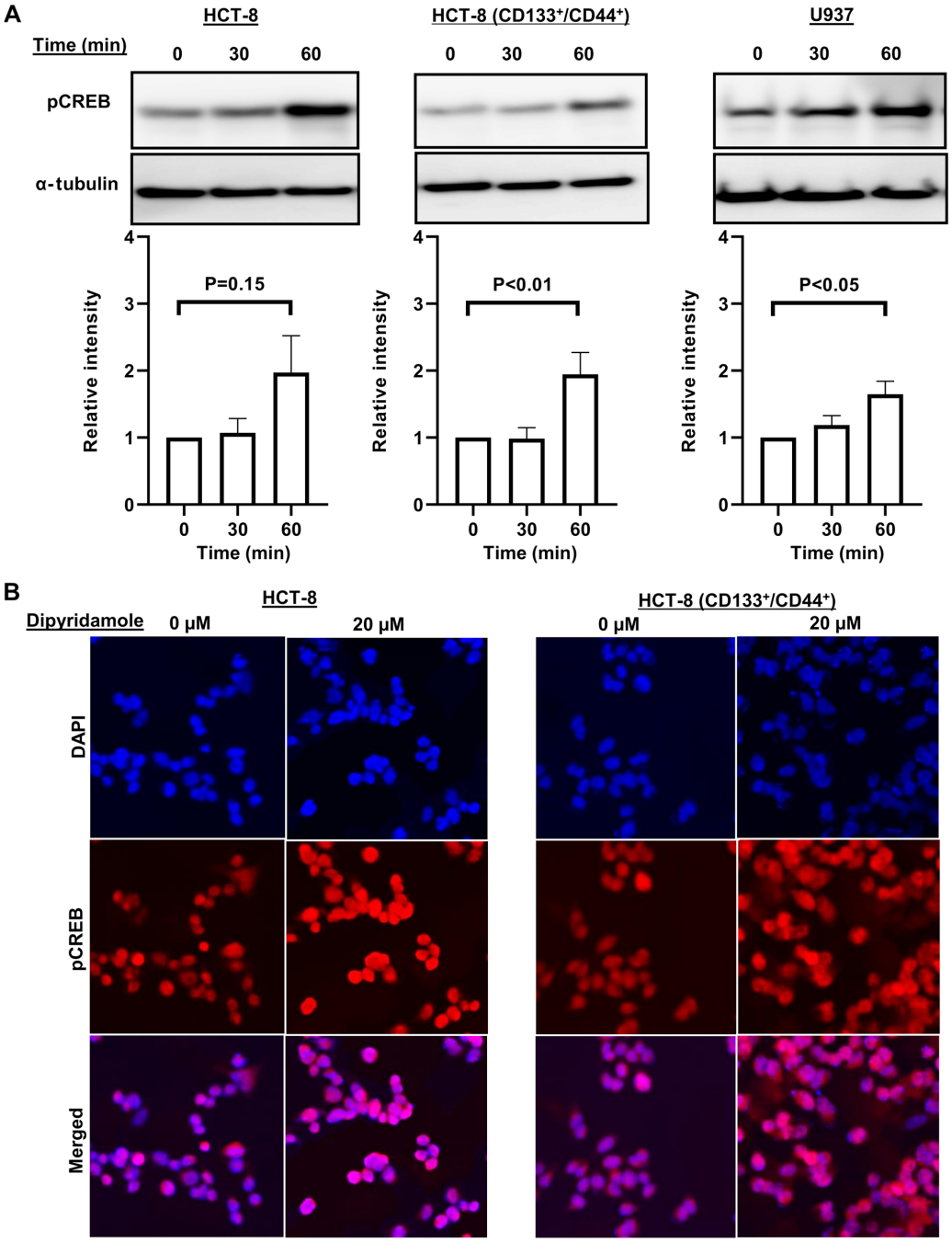

Figure 2. Expression of pCREB in HCT-8 cells, $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells and U937 cells. (A) Western blot analysis of the expression of pCREB in cells treated with 20 μM dipyridamole. (B) Immunofluorescence staining of the expression of pCREB in cells 60 min after treatment with 20 μM dipyridamole (magnification, x60). pCREB, phosphorylated cAMP-regulatory element-binding protein.

and proliferation of cancer cells. Thus, dipyridamole may promote, rather than inhibit, the survival and proliferation of cells, but further details of the relevant mechanisms require additional investigations.

Doxorubicin is commonly used for *in vitro* experiments and clinics (21,25-30). However, doxorubicin is rarely prescribed to patients with colorectal cancer as the expression of P-glycoprotein in colorectal cancer contributes to doxorubicin resistance (23,26). As dipyridamole has been reported to inhibit P-glycoprotein (28), synergistic effects of dipyridamole and doxorubicin are expected for patients with colon cancer. The present study investigated whether dipyridamole could enhance the cytotoxicity of doxorubicin to these cancer cells. Interestingly, the cytotoxicity of doxorubicin was significantly enhanced by dipyridamole, only to HCT-8 cells in particular dose. A previous study also reported that dipyridamole alone or in combination with methotrexate failed to increase the cytotoxicity in leukemia cells (29). In fact, the small compound of dipyridamole not only inhibits PDE3 and PDE5, but also regulates multiple cell signaling pathways (2,7). Therefore, the anti-cancer effect of dipyridamole used alone or with doxorubicin may largely depend on the cell types and other conditions (30). Further basic experiments using additional cancer cell lines, as well as clinical

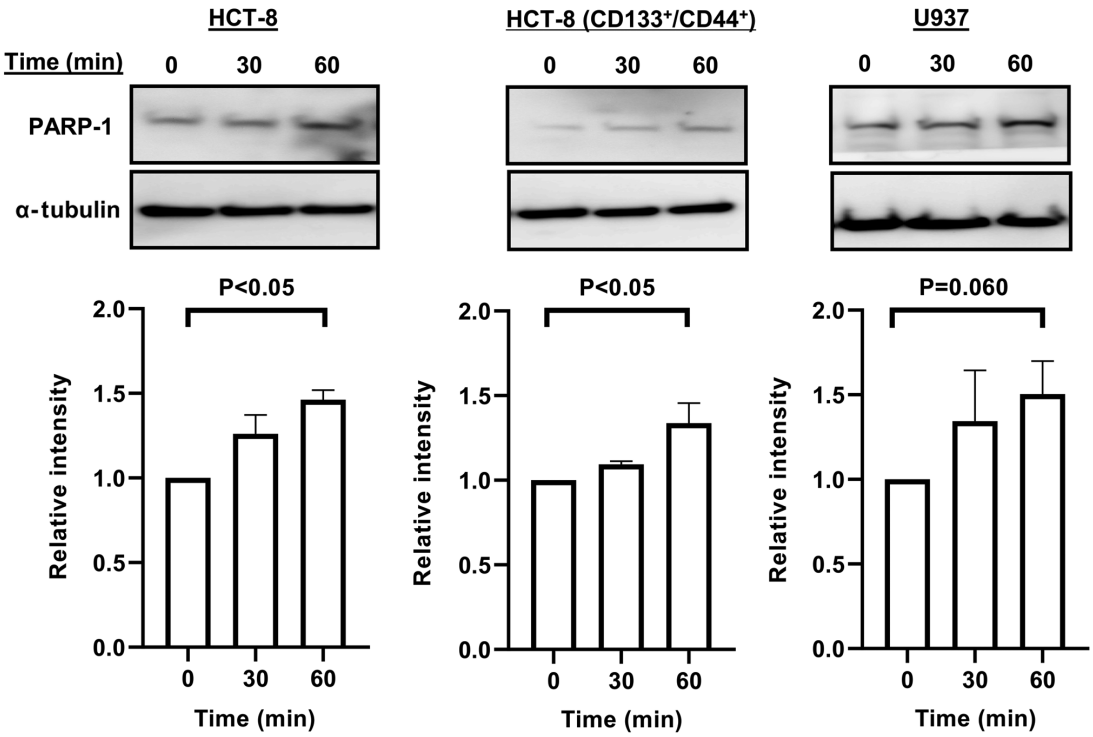

Figure 3. Expression of PARP-1 in HCT-8 cells, $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells and U937 cells treated with 20 μM dipyridamole. Representative images and semi-quantitative data of western blot analysis. PARP-1, poly(ADP-ribose) polymerase-1.

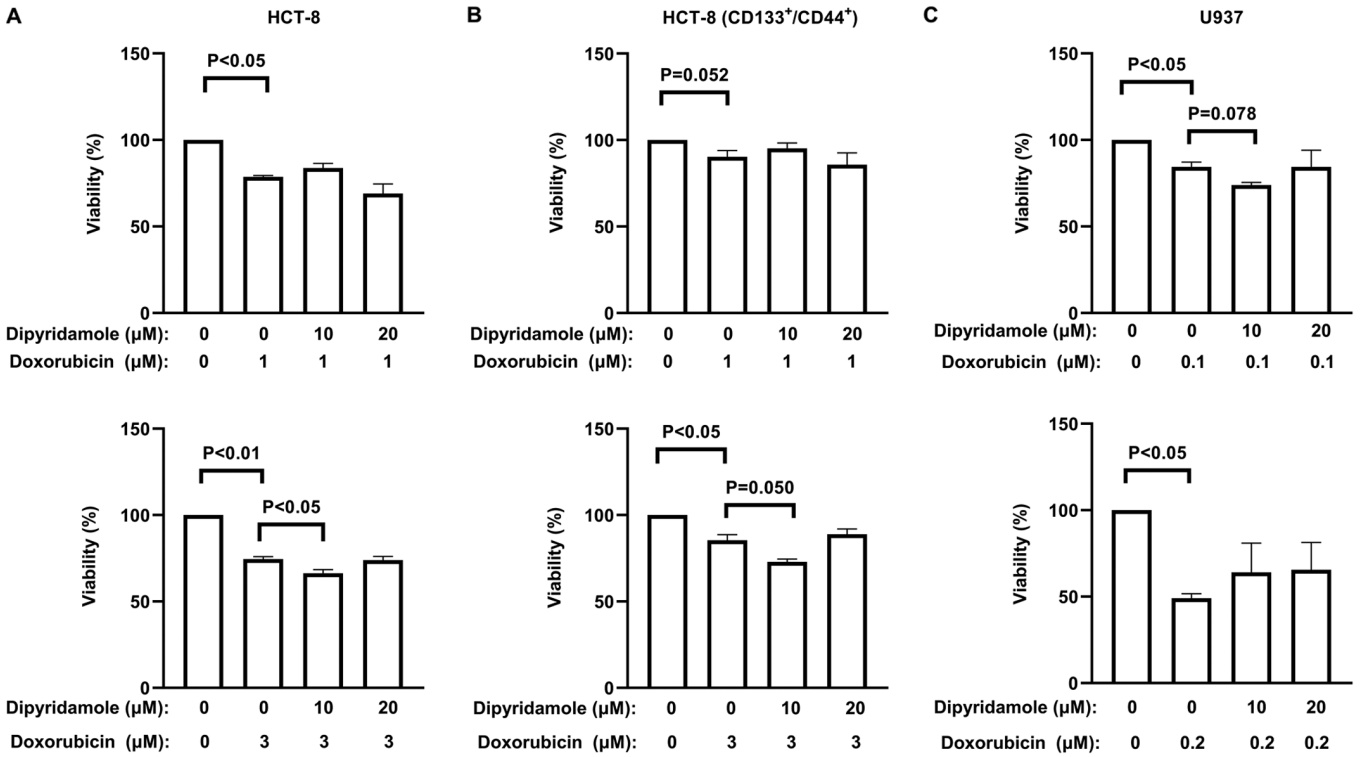

Figure 4. MTT assay was used to analyze the proliferation of (A) HCT-8 cells, (B) $CD133^+/CD44^+$ stem-like subpopulation of HCT-8 cells and (C) U937 cells. Cancer cells were treated with dipyridamole and doxorubicin at different doses.

data, are required to confirm the potential anti-cancer effect of dipyridamole.

In conclusion, to the best of our knowledge, the present preliminary data from *in vitro* experiments indicated for the first time that dipyridamole enhanced doxorubicin sensitivity at particular doses. As dipyridamole was also found to improve the survival and proliferation of cancer cells, it may be prescribed cautiously for patients with cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LA performed the experiments, acquired, analyzed and interpreted the data, and drafted the manuscript. NEM designed the current study and gave final approval for the manuscript to be published. TK and SG performed the experiments. TSL conceived the current study, performed the experiments and wrote and reviewed the final manuscript. TSL also gave final approval for the manuscript to be published and supervised the study. 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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