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ORIGINAL PAPER

Effect of Poly (ADP-ribose) Polymerase-1 Inhibition on the Proliferation of Murine Colon Carcinoma CT26 Cells

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Abstract To investigate effect of poly (ADP-ribose) polymerase inhibition on the proliferation of CT26 cells *in vitro* and the mechanism of this effect. CT26 cells were treated with a range of concentrations of 5-Aminoisoquinolin-1-one (PARP inhibitor) *in vitro*. MTT assays and flow cytometry were used to determine the proliferation and cell cycle distribution, respectively, of the cells. The expression of PARP-1 was investigated by Western blot. The binding of Nuclear Factor-κB to DNA was detected by electrophoretic mobility shift assay. The proliferation of CT26 cells was significantly inhibited by 5-AIQ induced PARP inhibition in a dose-dependent manner. Proliferation was inhibited by 17.5% at 100 μM 5-AIQ, by 27.6% at 500 μM 5-AIQ and by 39.9% at 1000 μM ($P<0.05$). After treatment with 5-AIQ, the proportion of cells in G_0/G_1 phases increased and the proportion of cells in S phase decreased. The expression of PARP-1 was attenuated in 5-AIQ-treated CT26 cells ($P<0.05$) and the binding of NF-κB to DNA binding was similarly diminished ($P<0.05$). These results suggest that PARP inhibition reduced the proliferation of CT26 cells *in vitro* and influences the cell cycle. This inhibition is mediated by inhibiting PARP-1, which then diminishes the activity of NF-κB.

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Abbreviations

PARP	Poly (ADP-ribose)polymerase
5-AIQ	5-Aminoisoquinolin-1-one
NF-κB	Nuclear Factor-κB
ICAM-1	intercellular adhesion molecule-1

Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme found in most eukaryotic cells, which builds polymers of ADP-ribose onto its substrate proteins by transferring ADP-ribose units from its substrate NAD^+. It plays an important role in regulating repair of damaged DNA repair, gene transcription, progression through the cell cycle and apoptotic cell death. Thus this enzyme is relevant to the genesis or development of various disease states. Several studies demonstrated that inhibitors of the catalytic activity of PARP-1 can reduce the tissue injury associated with ischaemia-reperfusion and the development of inflammation. These effects are mediated, at least in part, by lowering the activity of NF-κB causing inhibition of the up-regulation of expression of intercellular adhesion molecule-1 (ICAM-1) and P-selectin.[1–3] It has been reported that the expression of the PARP-1 protein is markedly higher in various malignant tumours, such as colon carcinoma, basal cell carcinoma, myeloma, carcinoma of the uterine and malignant lymphoma, than in the normal control tissues.[4–7] Our previous study also showed that the activity of PARP was significant higher in colorectal carcinoma than in the control colon mucosa.[8] 5-Aminoisoquinolin-1-one (5-AIQ) is a moderately potent and highly water-soluble inhibitor of PARP-1, which shows good activity in models of ischaemia-reperfusion

injury and of inflammation *in vitro* and *in vivo*. [1–3, 9]. Recently, a study reported [10] that inhibitors of PARP-1 catalytic activity, including 5-AIQ, reduced vascular endothelial growth factor-induced proliferation of human umbilical vein endothelial cells *in vitro* but there has been no report as to whether 5-AIQ can reduce the proliferation of tumor cells as a sole agent.

In this study, inhibition of the catalytic activity of PARP-1 with 5-AIQ was achieved in CT26 murine colon carcinoma cells. The effects of this inhibition on the proliferation and on the cell cycle were investigated, along with measurement of the NF-κB activity by EMSA.

Materials and Methods

Cell Culture

Murine colon carcinoma CT26 cells (kindly provided by Prof. Y. Q. Wei, Huaxi Hospital, Sichuan University) were grown in RPMI 1640 medium with the addition of 10% calf serum and standard concentrations of streptomycin and penicillin at 37°C in a 5% CO_2 incubator.

MTT Assays

Cells growing in log phase were plated at 3×10^4cells mL^{-1} in a 96-well plate in 10% RPMI 1640 medium as described above; 190 μL cell suspension was added to each plate. Four groups of cells were used in the experiment. The cells in groups one, two and three were treated with 5-AIQ [11] (100 μM, 500 μM and 1000 μM, respectively). The fourth group comprised cells untreated with 5-AIQ and served as a control group. Three wells were set up for each group. 5-AIQ was added to make up the final indicated concentration after the cells had been cultured for 10 h. A corresponding volume of normal saline was added to the control Group Four. After incubation for a further 20 h, the cells were treated with MTT (0.02 mL of a 5.0 mg mL^{-1} solution in PBS, Sigma) for 4 h. The media were removed after centrifugation and DMSO (150 μL) was added to each well. The absorbance (A) at 630 nm of each well was measured by the microplate reader. The experiment was repeated thrice. The inhibition proliferation of murine colon carcinoma CT26 cells induced by 5-AIQ was calculated as: inhibition ratio (%) = (1-(mean absorbance of treated group) / (mean absorbance of control group)) ×100%.

Cell Cycle Analysis

The cells were grown at an initial density of 3×10^4 cells mL^{-1} in 100 mL culture flask for 12 h, as previously described. 5-AIQ was then added to give final concentrations of 100 μM, 500 μM and 1000 μM in the experimental groups and NS was added to the control group. After a further 20 h, the cells were harvested, resuspended in PBS and fixed with cold 70% aqueous ethanol. The suspension of single cells was stained with PI (propidium iodide) and analysed with a flow cytometer (FCM, Becton Dickinson).

Western Blot Analysis for PARP-1

One control group (untreated with 5-AIQ) and one experimental group (treated with 5-AIQ at 100 μM) were set up for this experiment. The CT26 cells were grown at 3×10^4 cells mL^{-1} in a 100 mL culture flask for 12 h as previously described. Then 5-AIQ was added to give a final concentration of 100 μM in the experimental group and NS was added to in the control group. After 20 h, the cells were harvested. The nuclear extracts of CT26 cells were prepared according to the manufacturer's protocol for the kit (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents, Pierce). The concentrations of protein were determined by the Bradford method. [12] Nuclear extracts (20 μg) prepared from the cells were separated on 8% SDS-PAGE and transferred onto a PVDF membrane. The membrane was saturated by incubation at room temperature for 1 h with 5% (w/v) defatted dried milk in TTBS (Tris-buffered saline+0.1% (v/v) Tween-20) and then incubated at 4°C overnight with rabbit anti-mouse PARP polyclonal antibody (1:300, Santa Cruz) or with rabbit anti-mouse β-actin polyclonal antibody (1:300, Santa Cruz). The membranes were then washed thrice with TBST and incubated with peroxidase-conjugated goat anti-rabbit IgG at 37°C for 1 h. After having been washed with TBST again, the blots of the membranes were detected according to the manufacturer's protocols (ECL; Santa Cruz). Images were taken in a gel formatter (Bio-Rad).

Table 1 Inhibition of proliferation of CT26 cells *in vitro* by PARP inhibition

Groups	$A_{630}(\bar{x}\pm s)$	Percentage inhibition of proliferation
Control 5-AIQ-untreated	0.159±0.010	–
5-AIQ (100 μM)	0.132±0.006 *	17.5%
5-AIQ (500 μM)	0.114±0.009 *$^{\Delta}$	27.6%
5-AIQ (1000 μM)	0.094±0.012 * $^{\Delta}$**	39.9%

CT26 cells were treated with100 μM, 500 μM and 1000 μM of 5-AIQ. 5-AIQ-untreated CT26 cells are also shown. Proliferation of CT26 cells were determined as explained in MATERIALS AND METHODS. Values are means ±SE; n=9 observations per treatment group. *P<0.001, as compared to control; $^{\Delta}P$<0.001, as compared to 5-AIQ (100 μM); **P<0.001, as compared to 5-AIQ (500 μM)

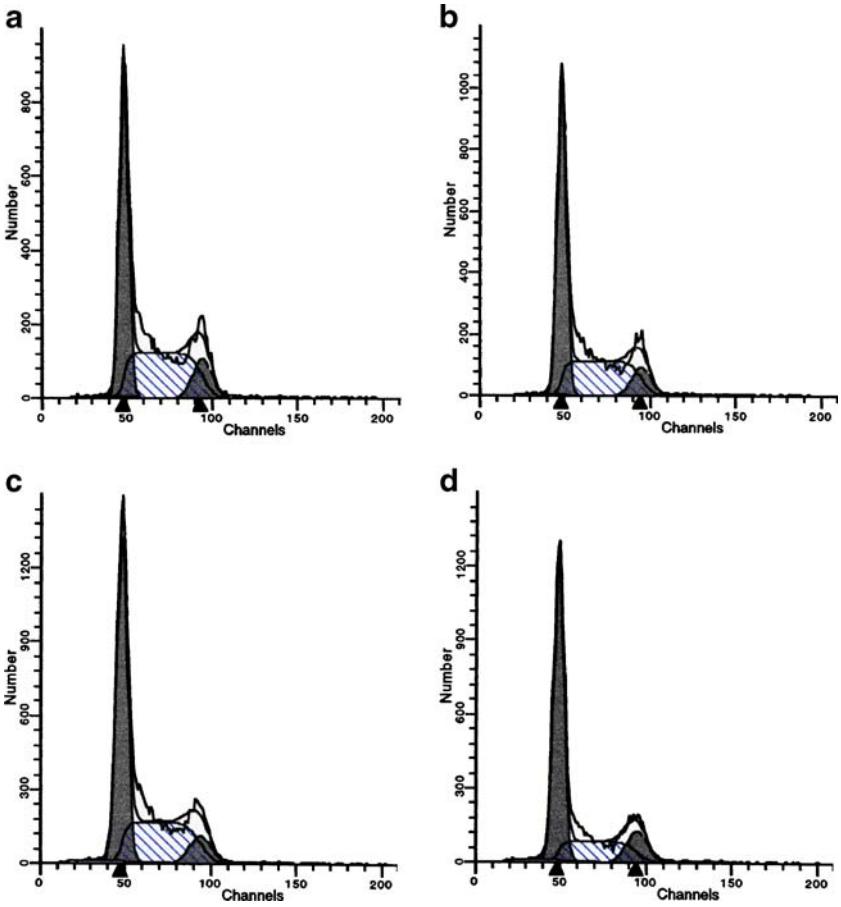

Fig. 1 The effect of PARP inhibition on the cell cycle of colon carcinoma CT26 cells analysed by FCM DNA per cell distribution histograms of cells stained with propidium iodide. Channel numbers represent the fluorescence intensity (arbitrary units), which in turn represents the amount of DNA per cell. A: 5-AIQ-untreated CT26 cells; B: 5-AIQ-treated (100 μM) CT26 cells; C: 5-AIQ-treated (500 M) CT26 cells; D: 5-AIQ-treated (1000 μM) CT26 cells. Note the two peaks: the left peak represents cells in the G0/G1 phase and the right peak represents cells in the G2+M phase

The integral optical density(IOD) ratio of the PARP-1 and β-actin bands was taken as a measure of expression of PARP-1 protein. The experiment was repeated thrice.

Electrophoretic Mobility Shift Assays (EMSA)

The cells were prepared as described as in the Western blot experiments above. The nuclear extracts of CT26 cells were prepared according to the manufacturer's protocol (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents, Pierce). The concentrations of protein were determined by the Bradford method. [13] EMSA were performed according to the manufacturer's protocol (LightShift™ Chemiluminescent EMSA Kit, Pierce). Nuclear extracts (3.0 μg) for each sample were incubated at room temperature for 20 min with reaction buffer containing 10 × Binding Buffer (2.0 μL), 50% aqueous glycerol (1.0 μL), $MgCl_2$ (100 mM, 1.0 μL), Poly-dI-dC (1.0 ng μL^{-1}, 1.0 μL), 1% NP-40 (1.0 μL) and biotin-labelled oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′, 2.0 μL (kindly provided by Prof. F.-C. Wang, College of Preventive Medicine, Third Military Medical University, China). Double-distilled water H_2O was used to make up the reaction volumes to 20 μL.The negative control was prepared by the reaction buffer above except the nuclear extracts. And the specificity of the DNA~protein binding was determined for NF-κB by a competition reaction in which a 50-fold molar excess of unlabelled oligonucleotide (kindly provided by Prof. F.-C. Wang, College of Preventive Medicine, Third Military Medical University, China) was added to the binding reaction. Protein~nucleic acid complexes were resolved by electrophoresis on 6% non-denaturing polyacrylamide gels at 100 V for 40 min. The gel bands were transferred to a nylon membrane (Sigma) using an electrical transfer apparatus. Subsequently, the DNA was cross-linked to the membrane by irradiation with UV light.Then the DNA was incubated in blocking solution (supplied with the LightShift kit), followed by incubation of the membrane with Light Shift™ Stabilized

Table 2 The effect of PARP inhibition on the cell cycle of colon carcinoma CT26 cells

Groups	Cell Cycle		
	G_0/G_1 (%)	S (%)	G2-M (%)
control (5-AIQ-untreated)	45.7	42.9	11.4
5-AIQ-treated (100 μM)	51.4	39.1	9.5
5-AIQ-treated (500 μM)	52.6	38.4	8.9
5-AIQ-treated (1000 μM)	61.5	25.8	12.7

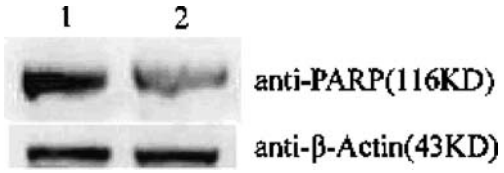

Fig. 2 The expression of PARP-1 in the nuclei of 5-AIQ-treated or untreated colon carcinoma CT26 cells CT26 cell were treated with and without 5-AIQ, and cell lysates were resolved by 8% SDS-PAGE and immunoblotted with anti-PARP-1 antibody. b- Actin served as the loading control.1: Control (5-AIQ-untreated); 2: 5-AIQ-treated (100 μM)

Streptavidin-Horseradish Peroxidase Conjugate.After extensive washing, reacting with substrate, signal was detected with chemiluminescence reagents(supplied with the kit). Finally, images were obtained of the membrane using a gel formatter (Bio-Rad). The IOD of protein~DNA binding complex was analysed by Quanlity one software. The relative density unit representing the NF-κB activity was obtained by comparing with the standard preparation [14]. The experiment was repeated thrice.

Statistical Analysis

The quantitative data were expressed as mean±SD ($\bar{x} \pm s$) Statistical analysis was performed by one-way ANOVA or Student's t test using SPSS 10.0 software. A p-value less than 0.05 was considered to be significant.

Results

Effect of PARP Inhibition on Cell Proliferation

The MTT assay was used to evaluate the inhibitory effect of 5-AIQ on the growth of CT26 cells *in vitro*. As can be seen in Table 1, the absorbance due to the MTT-derived formazan in every experimental group was lower than that in control group. These data indicate that 5-AIQ inhibits proliferation of the CT26 cells in a dose-dependent manner (Table 1).

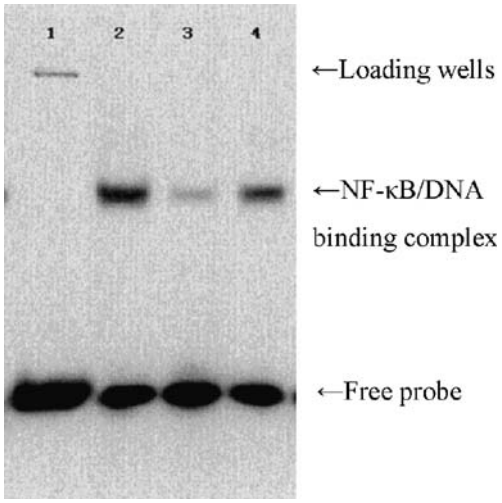

Fig. 3 The effect of PARP inhibition on binding of NF-kB to DNA in colon carcinoma CT26 cells Electrophoretic mobility shift assay was performed with nuclear extracts of CT26 cells treated with and without 5-AIQ. The results shown are representative of three independent experiments. Lane 1 negative control (reaction buffer only), Lane 2 5-AIQ-untreated, Lane 3 specificity control, Lane 4 5-AIQ-treated (100 μM)

Effect of PARP inhibition on the cell cycle

Figure 1 and Table 2 show the distributions of each group of murine colon carcinoma CT26 cells in the phases of the cell cycle, as shown by flow cytometry. The cell cycle dispositions were different between the experiment groups and the control group. The proportion of cells in G_0/G_1 phase in each 5-AIQ-treated group was higher than that in the control group (5-AIQ-untreated), in a dose-dependent manner. In addition, the proportion of cells in S phase in each 5-AIQ-treated group was lower than that in the control group, in a dose-dependent manner.

Effect of 5-AIQ on the Amount of PARP-1 Protein

The amount of PARP-1 protein in the 5-AIQ-treated groups was clearly less than that in the control group (5-AIQ-untreated) ($p<0.05$), as shown in Fig. 2 and Table 3.

Table 3 The expression of PARP-1 in the nuclei of 5-AIQ-treated or untreated colon carcinoma CT26 cells

Groups	PARP(ratio)
control (5-AIQ-untreated)	1.261±0.195
5-AIQ-treated (100 μM)	0.828±0.096*

CT26 cells were treated with100 μM of 5-AIQ. 5-AIQ-untreated CT26 cells are also shown. PARP-1 expressions of CT26 cells were determined as explained in MATERIALS AND METHODS.Values are means ±SE; n=3 observations per treatment group. *$P<0.05$, as compared to control

Table 4 The change of NF-κB binding activity in 5-AIQ-treated or untreated colon carcinoma CT26 cell groups

Groups	NF-κB activity (RDU
Control (5-AIQ-untreated)	3334±435
5-AIQ-treated-(100 μM)	1941±424*

CT26 cells were treated with100 μM of 5-AIQ. 5-AIQ-untreated CT26 cells are also shown. NF-κB binding activity of CT26 cells were determined as explained in MATERIALS AND METHODS.Values are means ±SE; n=3 observations per treatment group. *$P<0.05$, as compared to control

Effect of PARP Inhibition on Binding of NF-κB to DNA

Only free probe could be detected in the negative control lane, with no NF-κB~DNA binding complex (Fig. 3, Lane 1) A band corresponding to the NF-κB~DNA complex was clearly evident in the 5-AIQ-untreated control (Lane 3) and the specificity of the binding was demonstrated by a very large reduction in the intensity of this band in the presence of the unlabelled DNA oligomer (Lane 2). The integral optical density of NF-κB~DNA binding complex in 5-AIQ-treated group (100 μM) (Lane 4) was significantly weaker than that in the control group (5-AIQ-untreated) (p=0.017) (see Table 4).

Discussion

5-AIQ is a potent, highly water-soluble inhibitor of the catalytic activity of PARP-1 [3], playing an important role ameliorating the tissue injury associated with ischaemia-reperfusion and inflammation. Rajesh *et al*. [10] demonstrated that 5-AIQ reduced the proliferation of human umbilical vein endothelial cells induced by vascular endothelial growth factor *in vitro* but there is no previous report on the effect of this agent on the proliferation of tumor cells. Our results show, for the first time, that 5-AIQ, albeit at a high dose (100–1000 μM), reduces the proliferation of murine colon carcinoma CT26 cells in a dose-dependent manner (p<0.05). FCM shows that more cells were in G_0/G_1 phase and less in S phase in each 5-AIQ-treated group than in the control group (5-AIQ-untreated), also in a dose-dependent manner. These data suggest that proliferation was reduced owing to 5-AIQ (and thus inhibition of PARP-1) causing a block at the $G_1 \rightarrow S$ transition of the cell cycle.

Rajesh *et al*. [10] showed that 5-AIQ reduced vascular endothelial growth factor- or fibroblast growth factor-induced proliferation of human umbilical vein endothelial cells by inhibiting the activity of PARP-1. Inhibition of PARP-1 with 5-AIQ has also been reported to reduce the expression of adhesion molecules such as ICAM-1 and P-selectin in organs such as colon, liver and lung injury, consequent to ischaemia-reperfusion and acute inflammation; this effect is mediated through reduction of the activity of NF-κB. [1–3]. Another experiment indicated that PARP could bind to NF-κB to form a complex, then the DNA binding site of NF-κB was exposed and transposed into cell nucleus, then the transcription of NF-κB-dependent genes such as cell adhesion molecule was up-regulated and the expressions of the protein were increased. Genovese et al. [15] showed that treatment with 5-AIQ reduced the development of inflammation and tissue injury events associated with spinal cord trauma though inhibiting PARP-1 catalytic activity, thus inhibiting the DNA-binding activity of NF-κB. Our previous study [16] also demonstrated that 5-AIQ inhibited the activity of PARP-1 in human colon carcinoma HT29 cells and reduced the expression of NF-κB-dependent genes such as ICAM-1 and P-selectin. However, it is known that NF-κB plays an important role in the genesis and development of colorectal carcinomas [17–19]. It can promote the genesis of colorectal carcinomas by regulating oncogenes, such as *c-myc*, or increase proliferation by up-regulating cyclooxygenase-2. Moreover, it can promote the metastasis of colorectal carcinomas by up-regulating the expressions of ICAM-1, vascular endothelial growth factor and Matrix Metalloproteinase-2.[15, 16, 20, 21] In the present study, Western Blot analysis also showed that the amount of PARP-1 protein in the nuclei of 5-AIQ-treated colon carcinoma CT26 cells was lower than that in 5-AIQ-untreated group. Interestingly, this suggests that, at high concentrations, 5-AIQ can not only inhibit PARP-1 activity but also the amount of the PARP-1 protein in the nuclei of CT26 cells. Since we have also shown that inhibition of PARP-1 activity with 5-AIQ decreases the amount of the NF-κB~DNA binding complex in CT26 cells, compared to the control group (p<0.05), it is need to be further studied that if synthesis of PARP-1 protein is controlled by NF-κB.

Taken together, our results demonstrate that 5-AIQ can inhibit the proliferation of murine colon carcinoma CT26 cells. This effect is consequent to 5-AIQ inhibiting PARP-1 activity. PARP-1 may play a role in the proliferation of colon carcinoma. The detailed mechanism of these processes will be the subject of further studies.

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