Poly(ADP-Ribose) Polymerase Inhibition Down-Regulates Expression of Metastasis-Related Genes in CT26 Colon Carcinoma Cells

Ming Li^a Michael D. Threadgill^b Yalan Wang^a Li Cai^a Xiao Lin^a

^aDepartment of Pathology, Chongqing Medical University, Chongqing, People's Republic of China;

^bDepartment of Pharmacy and Pharmacology, University of Bath, Bath, UK

Key Words

Cell adhesion • Colorectal cancer • Metastasis • Nuclear factor- κ B • PARP inhibitors • Poly(ADP-ribose) polymerase

Abstract

Objectives: The current study was designed to test the hypothesis that inhibition of poly(ADP-ribose) polymerase in colorectal cancer mediates down-regulation of metastasis-related gene expression through the regulation of nuclear factor- κ B (NF- κ B) activity. **Methods:** Mouse colon carcinoma cells (CT26) were treated with and without the PARP inhibitor 5-aminoisoquinolin-1(2H)-one hydrochloride (5-AIQ). We investigated adhesion, migration and invasion of differently treated CT26 cells. In addition, the expression levels of PARP, NF- κ B, integrin β_1 , MMP-9 and MMP-2 as well as the activities of NF- κ B, MMP-9 and MMP-2 were determined by Western blot, electrophoretic mobility gel shift assay and zymography, respectively. **Results:** Inhibition of PARP attenuated the adhesion of CT26 cells to the extracellular matrix and their migration and invasion through Matrigel. In addition, the results of Western blot showed that the expression levels of PARP, NF- κ B, integrin β_1 , MMP-9 and MMP-2 were reduced in 5-AIQ-treated CT26 cells; the activities of NF- κ B, MMP-9 and MMP-2 were also suppressed. **Conclusions:** Inhibition of PARP down-regulates the expression of metastasis-related genes in mouse colon carcinoma cells. This could be, at least in part, through the regulation of NF- κ B activity, but the precise mechanisms of action remain to be elucidated.

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Introduction

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme of eukaryotic cells. When activated by DNA single-strand breaks, PARP uses ADP-ribose units from its substrate NAD⁺ to build a polyanionic poly(ADP-ribose) polymer onto Glu residues on its target proteins, which can include histones and PARP itself (automodification) [1, 2]. The formation of this polymer then triggers recruitment of the repair apparatus to repair the breaks. Thus PARP controls the repair of DNA damaged by environmental agents and by cancer therapeutic agents such as DNA-targeted cytotoxins (e.g. alkylating agents and topoisomerase II inhibitors) and ionizing radiation (radiotherapy). The role of inhibitors of PARP as potentiators of chemotherapy and radiotherapy has been studied for many years, and several drugs have been evaluated in clinical trials [3–6]. Very recently, pharmacological inhibition of PARP has been proposed as monotherapy in BRCA mutant tumors [7].

In normal cells, excessive DNA damage causes over-activation of PARP, which, in turn, results in rapid depletion of the intracellular NAD⁺ and ATP, eventually leading to cellular dysfunction and death [8]. This is particularly evident in ischemia-reperfusion disorders, where inhibition of PARP activity can lead to protection against

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Yalan Wang
Department of Pathology
Chongqing Medical University
Chongqing 400016 (People's Republic of China)
Tel. +86 23 6848 5789, Fax +86 23 6848 5111, E-Mail wangyalan074@126.com

cell death and organ damage in models of hemorrhagic shock, myocardial infarction, and ischemic kidney disease and ischemic liver disease, inter alia [9–13]. More recently, overactivation of PARP has been shown to be involved in the regulation of inflammatory processes, with some inhibitors of PARP showing anti-inflammatory effects in animal models, particularly those of acute inflammation [14–18]. This pro-inflammatory effect of PARP is associated with some transcription factors, particularly nuclear factor (NF)- κ B [19–21]. A recent report has shown that PARP inhibitors affect NF- κ B activation and gene expression via the activation of the PI₃-kinase/Akt (protein kinase B) pathway, which can inhibit mitogen-activated protein kinase activation for example [22]. On the other hand, automodification of PARP by poly(ADP-ribosylation) up-regulates the formation of the NF- κ B-DNA complex and enhances the NF- κ B-dependent expression of the genes for some inflammatory mediators [23].

Inhibitors of PARP have long been proposed as potentiators of DNA-damaging chemotherapy and of radiotherapy through inhibition of repair of damaged DNA, but only a few papers show overexpression of PARP in tumors, e.g. colon cancer [24], human cervical cancer [25] and prostate cancer [26]. PARP has recently been implicated in the processes of angiogenesis, interestingly including control of expression of HIF-1 α and thus vascular endothelial growth factor [27], and some inhibitors of the enzymatic activity of PARP show anti-angiogenic effects [28]. We have previously demonstrated that inhibition of PARP activity with 5-aminoisoquinolin-1(2H)-one hydrochloride (5-AIQ) suppresses the expression of P-selectin and intercellular adhesion molecule-1 (ICAM-1) in vivo in an animal model of lung injury [16] and in vitro in human colon cancer cells [29]. These data suggest that, in addition to chemo- and radiosensitization, inhibitors of PARP may have other roles in the treatment of cancer.

5-AIQ is a highly water-soluble inhibitor of PARP activity [30]. In vitro, it is moderately potent and highly selective for inhibition of PARP activity compared with the inhibition of the mono(ADP-ribosyl)ation activity of diphtheria toxin [30], and is exquisitely potent in vivo in protecting against ischemia-reperfusion-triggered organ damage in a rodent model of hemorrhagic shock [9]. 5-AIQ can reduce ischemia/reperfusion injury of the liver [13] and has been shown to provide beneficial effects in rodent models of heart transplantation [31], spinal cord injury [15] and acute lung injury [16]. Notably, 5-AIQ inhibits neutrophil-mediated lung injury through down-

regulation of the expression of P-selectin and ICAM-1 [16]. However, the effects of 5-AIQ (and other PARP inhibitors) on the NF- κ B-mediated invasive aspects of tumor metastases are unknown. It is known that integrins mediate the adhesion of cancer cells to the extracellular matrix (ECM) [32]; additionally, matrix metalloproteinase-9 (MMP-9) and -2 (MMP-2) play a major role in the degradation of type-IV collagen [33], which is the major component of ECM. All of them play an important role in tumor metastasis. Therefore, in this study, we investigated the role and mechanism(s) by which pharmacological inhibition of PARP with 5-AIQ may lead to the down-regulation of NF- κ B, integrin β_1 , MMP-9 and MMP-2, thereby inhibiting adhesion of mouse colon carcinoma cells (CT26) to ECM and invasion through Matrigel.

Materials and Methods

Cell Culture

CT26 cells were a kind gift from Prof. Wei Yuquan (Sichuan University, China); they were cultured in RPMI 1640 (Invitrogen, Gibco, Calif., USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU ml⁻¹) and 100 μ g ml⁻¹ streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. For each experiment, subconfluent cells in culture flasks were used after trypsinization.

Adhesion Assay

Cell adhesion was assayed as described by Odero-Marrah et al. [34]. Briefly, 96-well culture plates were precoated with 50 μ l of a serially diluted fibronectin (30 μ g ml⁻¹; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) solution and air-dried overnight at 4°C. The plates were then blocked with bovine serum albumin (10 mg ml⁻¹) in phosphate-buffered saline (PBS) at 37°C for 2 h. CT26 cells were pretreated for 16 h with or without 5-AIQ (100 μ M) before being resuspended in serum-free medium. Cells (2.5×10^5 ml⁻¹) were seeded to wells coated with fibronectin (200 μ l well⁻¹) and incubated at 37°C for 30 min. Non-adherent cells were removed by gentle washing with PBS. Cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2.0 mg ml⁻¹, 50 μ l well⁻¹) at 37°C for 4 h; dimethylsulfoxide (150 μ l well⁻¹) was then added and the plates were oscillated for 10 min to solubilize the formazan crystals. The absorbance of each well was measured in a microplate reader (Labsystems) at 630 nm.

The results are presented as percentage attachment, where 100% attachment corresponds to the attachment of untreated cells exposed to fibronectin.

Migration and Invasion Assay

Migration and invasive abilities were measured in Transwell cell culture chambers (Costar) according to the method of Wick et al. [35], with a slight modification. In brief, the lower surface of the membrane was coated with fibronectin (Santa Cruz Biotechnology; 5.0 μ g well⁻¹) and then air-dried overnight at room temperature. CT26 cells, pretreated for 16 h with or without 5-AIQ

(100 μM), were resuspended in serum-free RPMI 1640. Cells at a density of $1 \times 10^6 \text{ ml}^{-1}$ were seeded in each upper chamber with serum-free RPMI 1640 medium (100 μl). RPMI 1640 medium with 10% FBS (600 μl) was added to the lower chambers as a chemoattractant. At least three chambers were used for each experimental group. After incubation for 24 h in a humidified CO_2 incubator at 37°C , the inserts were fixed with methanol and stained with hematoxylin. The cells on the upper surface of the membrane were removed using cotton swabs. The cells on the lower side of membrane were counted by light microscopy in five fields (up, down, left, right and middle) at a magnification of $\times 400$.

For the invasion assay, the upper surface of the membrane (6.5-mm diameter and 8- μm pore size) was coated with Matrigel (diluted 1:5 with serum-free RPMI 1640 medium; Becton Dickinson, Bedford, Mass., USA) at 30 $\mu\text{l well}^{-1}$. The procedure then followed the migration assay as above.

The results are presented as percentage invasion, where 100% invasion corresponds to the invasion of untreated cells.

Western Blot Analysis

CT26 cells were pretreated with or without 5-AIQ (100 μM) for 16 h, then washed with ice-cold PBS and lysed with M-PER[®] Mammalian Protein Extraction Reagent (Pierce), following the manufacturer's instructions. The nuclear proteins were extracted with NE-PER[®] nuclear and cytoplasmic extraction reagents (Pierce), following the manufacturer's instructions. Western blot analyses were carried out using procedures described by Lee et al. [36]. The protein concentrations from lysates of treated or untreated CT26 cells were determined by the Bradford protein assay. Equal amounts of protein (10–30 μg) were subjected to electrophoresis in SDS-polyacrylamide gels (8%) and were transferred to a polyvinylidene fluoride membrane. After blocking nonspecific binding sites, membranes were incubated with appropriate antibodies against PARP-1, NF- $\kappa\text{Bp}65$, integrin β_1 , MMP-9, MMP-2 and actin (diluted 1:200–1:500; Santa Cruz Biotechnology) overnight at 4°C . The immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Santa Cruz Biotechnology).

Electrophoretic Mobility Gel Shift Assay (EMSA)

CT26 cells were pretreated with or without 5-AIQ (100 μM) for 16 h. Nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce), following the manufacturer's instructions. Equal amounts of nuclear protein extracts were assayed for binding of NF- κB protein to DNA using the LightShift chemiluminescence EMSA kit (Pierce) with a biotin-end-labeled NF- κB oligonucleotide (5'-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3'; a gift from Dr. Wang Fengchao, Third Military Medical University of China). Assay mixtures that contained nuclear protein extracts (3 μg), reaction buffer [Tris (10 mM), KCl (50 mM), dithiothreitol (1.0 mM), glycerol (2.5%), MgCl_2 (5.0 mM), Nonidet P-40 (0.05%), poly(dI-dC) (1.0 μg)] and biotin-end-labeled NF- κB oligonucleotides (2 μl) were incubated in a final volume of 20 μl for 20 min at room temperature to allow binding of protein to DNA. Specific binding was confirmed by adding unlabeled probe (a gift from Dr. Wang Fengchao; 2 μl) as a specific competitor. Protein-DNA complexes were resolved on non-denaturing 6% polyacrylamide gels in $0.5 \times \text{TBE}$, transferred to nylon membranes via electroblotting and UV cross-

linked. The DNA-protein complexes were visualized with streptavidin-horseradish peroxidase followed by measurement of the chemiluminescence. For the supershift assay, the binding reaction was carried out in the presence of an anti-NF- $\kappa\text{Bp}65$ antibody (Santa Cruz Biotechnology).

Zymography

Equal numbers of CT26 cells were pretreated with or without 5-AIQ (100 μM) for 16 h, and gelatinase activities were detected as described by Quesada et al. [37]. The culture medium was collected and centrifuged at 14,000 rpm for 5 min at 4°C to remove cell debris. The supernatant was mixed with $4\times$ nonreducing sample buffer (4:1 v/v) and electrophoresed on a 10% SDS-polyacrylamide gel containing gelatin (0.1%; Sigma) as a substrate for MMP-2 and MMP-9. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h to remove SDS and then incubated overnight at 37°C in developing buffer [Tris (50 mM), HCl (40 mM), NaCl (200 mM), CaCl_2 (5.0 mM) and Brij (0.2%)]. After incubation, gels were stained with Coomassie brilliant blue (0.5%) in methanol (40%), acetic acid (10%) and water (50%) for 30 min followed by destaining. Gelatinolytic activity manifested as horizontal white bands on a blue background.

Statistical Analysis

All results were expressed as the mean $\pm$ SD. Student's *t* test was used to determine the significance ($p < 0.05$) of the differences between the mean values.

Results

PARP Inhibition Attenuates the Adhesion of CT26 Cells to ECM and Their Migration and Invasion

To determine whether PARP inhibition affects the integrin-mediated cancer cell adhesion to ECM, CT26 cells were incubated with or without the PARP inhibitor 5-AIQ and then their ability to adhere to fibronectin-coated plates was determined. Treatment of CT26 cells with this agent led to a significantly decreased adhesion of the cells to this matrix compared with a control group (fig. 1a).

To examine the role of PARP on the migration and invasion of CT26 cells, we used a Transwell chamber assay to examine the migratory and invasive potentials of CT26 cells pretreated with 5-AIQ in comparison with cells without this pretreatment (fig. 2). In this migration assay, the 5-AIQ-pretreated cells showed a decrease of 30% in the level of penetration through the microporous membrane compared with penetration of the CT26 cells which had received no drug (fig. 1b). Coincidentally, the number of permeating 5-AIQ-pretreated CT26 cells was decreased by 37% in Matrigel-coated Transwell chambers compared with the number of permeating 5-AIQ-untreated CT26 cells (fig. 1c). All these data showed that

Fig. 1. PARP inhibition suppresses CT26 cell adhesion to the ECM, and their migration and invasion. Control = 5-AIQ-untreated group; 5-AIQ = 5-AIQ-treated group. **a** Adhesion of CT26 cells was suppressed in 5-AIQ-treated cells compared with 5-AIQ-untreated (control) cells. The relative adhesion rate was calculated and normalized to the 5-AIQ-untreated (control) cells, set at 100 (n = 5). **b, c** In the migration and invasion assays, 5-AIQ

treatment resulted in low cell penetration through the Transwell membrane compared with 5-AIQ-untreated (control) cells. Relative migration and invasion rates were calculated as the mean number of cells in five fields, and normalized to the 5-AIQ-untreated (control) cells, set at 100 (n = 15). Means (columns) and SD (bars) of each group. * p < 0.05, ** p < 0.01, vs. control (Student's t test).

Fig. 2. The effects of PARP inhibition on migration and invasion of CT26 cells (Transwell chamber). Staining with hematoxylin; magnification, $\times 400$. Control (**a, c**; 5-AIQ untreated) and 5-AIQ-treated CT26 cells (**b, d**) in the migration (**a, b**) and in the invasion assays (**c, d**).

PARP inhibition contributes to a potentially anti-metastatic effect in CT26 cells.

PARP Inhibition Decreases NF- κ B Expression and Binding of NF- κ B to DNA

NF- κ B is an important factor in tumor metastasis, owing to its control of the expression of several metasta-

sis-related genes [38]. Expression of NF- κ B was determined in CT-26 cells (pretreated with 5-AIQ and non-pretreated) by Western blot analysis, which showed a significant decrease in the amount of the NF- κ B protein in the 5-AIQ-treated cells compared to the 5-AIQ-untreated cells (fig. 3). Some decrease in the amount of PARP-1 protein was also observed (fig. 3). In parallel, the binding

Fig. 3. PARP inhibition decreased expression of PARP and NF-κB by Western blot analysis. **a** PARP and NF-κBp65 expression in 5-AIQ-untreated (control) and 5-AIQ-treated CT26 cells. The actin protein was used as an internal loading control. **b** PARP and NF-κBp65 expression was decreased in 5-AIQ-treated cells compared with 5-AIQ-untreated (control) cells. They were calculated as the relative degradation and normalized to the 5-AIQ-untreated (control) cells, set at 100. Means (columns) and SD (bars) of each group (n = 3) are shown. * p < 0.05, vs. control (Student's t test).

Fig. 4. PARP inhibition decreased binding of NF-κB to DNA. **a** EMSA analysis was carried out for 5-AIQ-untreated (control) and 5-AIQ-treated CT26 cells. Lane 1 = 5-AIQ-treated group; lane 2 = specificity control; lane 3 = 5-AIQ-untreated group; lane 4 = negative control (reaction buffer only). **b** Binding of NF-κB to DNA was decreased in 5-AIQ-treated cells compared with 5-AIQ-untreated (control) cells. They were calculated as the relative degradation and normalized to the 5-AIQ-untreated (control) cells, set at 100. Means (columns) and SD (bars) of each group (n = 3) are shown. * p < 0.05, vs. control (Student's t test).

of NF-κB to DNA in 5-AIQ-treated and -untreated cells was assessed by gel shift assay. Inhibition of PARP by 5-AIQ significantly decreased the DNA-binding activity of NF-κB (fig. 4). These results indicate that PARP inhibition can decrease the expression of NF-κB and also the ability of NF-κB to bind to DNA.

PARP Inhibition Down-Regulates the Expression of Metastasis-Related Genes and, Consequently, the Enzymatic Activities of MMPs

The metastatic process requires the expression of several metastasis-related genes. In particular, integrin-β₁-mediated cell-matrix adhesion and MMPs are critical factors for the invasion of tumor cells into surrounding tissue [39–42]. In the present work, Western blot analysis was used to measure the expression of these metastasis-related genes and showed that the amounts of integrin β₁, MMP-9 and MMP-2 proteins significantly decreased by 45, 28 and 36.2%, respectively, in 5-AIQ-treated cells compared with 5-AIQ-untreated (control) cells (fig. 5). Gelatine zymography then showed that PARP inhibition following pretreatment with 5-AIQ leads to a decrease in the proteolytic activities of MMP-2 and MMP-9 compared with CT26 cells without 5-AIQ pretreatment (fig. 6), indicating that inhibition of PARP induces suppression of expression of important metastasis-related genes.

Discussion

PARP is involved in many cellular processes, including the control of repair of damaged DNA (especially single-strand breaks) and the control of gene expression/transcription. However, the involvement of PARP in tumor metastasis was unclear. In this study, we have shown

Fig. 5. PARP inhibition decreased the expression of integrin β_1 , MMP-2 and MMP-9 (Western blot analysis). **a** Expression of integrin β_1 , MMP-2 and MMP-9 in 5-AIQ-untreated (control) and 5-AIQ-treated CT26 cells. The actin protein was used as an internal loading control. **b** Integrin β_1 , MMP-2 and MMP-9 expression was decreased in 5-AIQ-treated cells, vs. control. They were calculated as the relative degradation and normalized to the 5-AIQ-untreated (control) cells, set at 100. Means (columns) and SD (bars) of each group (n = 6) are shown. * p < 0.05, ** p < 0.01, vs. control (Student's t test).

the following: (a) PARP inhibition by 5-AIQ decreases adhesion, migration and invasion of CT26 mouse colon adenocarcinoma cells; (b) PARP inhibition reduces expression of NF- κ B and its DNA-binding activity; (c) PARP inhibition decreases the expression of important metastasis-related genes (integrin β_1 , MMP-2 and MMP-9) and correspondingly diminishes the proteolytic activities of these MMPs in these cells. Taken together, these results point to PARP inhibition being an effective approach to inhibition of NF- κ B activity and down-regulation of expression of its dependent genes, such as integrin β_1 , MMP-2 and MMP-9, resulting in the inhibition of tumor metastases. Curiously, inhibition of PARP enzymatic activity also appeared to decrease the amount of PARP protein present, although the mechanism of this decrease is unclear (increased rate of degradation or diminution of expression of PARP protein possibly caused by decreased NF- κ B activity).

Metastasis occurs by a series of steps, including degradation of basement membranes, degradation of the stromal ECM and adhesion of cells to the ECM. The integrins mediate the adhesion between tumor cells and the ECM and the MMPs degrade the ECM. Together, they are considered to be amongst the most important factors in tumor metastasis. Activation of NF- κ B leads to up-regulation of several NF- κ B-dependent genes, including integrin β_1 [43], MMP-2 [44] and MMP-9 [45, 46], which are key to metastases. PARP has been reported to be a co-activator of NF- κ B in many inflammatory disorders [20,

Fig. 6. PARP inhibition decreased enzymatic activities of MMP-2 and MMP-9 in 5-AIQ-treated cells compared with 5-AIQ-untreated (control) cells (zymography). Means (columns) and SD (bars) of each group (n = 5) are shown. * p < 0.05, ** p < 0.01, vs. control (Student's t test).

47–49]; PARP acts synergistically with p300 and plays an essential regulatory role in NF- κ B-dependent gene expression [21]. Indeed, it has been proposed that acetylation of PARP by p300/CREB-binding protein is responsible for up-regulation of NF- κ B by PARP and that this

activating acetylation is reversed by histone deacetylases 1–3 [50]. In the context of inflammation, pharmacological inhibition of PARP has been demonstrated to down-regulate inducible nitric oxide synthase (iNOS), interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) [51, 52] through inhibition of expression and function of NF- κ B. Similarly, Zheng et al. [47] related control of expression of ICAM-1 to expression and function of NF- κ B and thus to control by PARP. Thus, the inhibition of PARP results in lower NF- κ B activity and lower expression of dependent genes. Therefore, it can be hypothesized that PARP-induced cell adhesion, migration and invasion are, at least in part, due to activation of the NF- κ B pathway.

Integrins are very important to cell-matrix adhesion in the processes of tumor metastasis. Moreover, integrin β_1 has been reported to be one of the most critical integrin subunits in the process of adhesion of CT26 cells to fibronectin, and CT26 cells demonstrate a chemotactic response towards this matrix protein [53]. In this study, consistent with our adhesion data, we found a significant reduction in the expression of integrin β_1 by pharmacological inhibition of PARP. We also showed that PARP inhibition reduced expression and DNA-binding activity of NF- κ B and concomitantly diminished the expression and proteolytic activity of MMP-2 and MMP-9. In parallel, we found that inhibition of PARP inhibited the invasive capability of CT26 colon cancer cells. MMPs have been implicated in metastases because of its role in the degradation of basement membrane collagen [21, 53]. Koh et al. [19] have already shown that PARP inhibition

with the relatively less potent inhibitor 3-aminobenzamide diminishes the expression of the MMP-9 protein and infiltration of neutrophils in a rat model of ischemic stroke. Similarly, Kauppinen and Swanson [49] have suggested that PARP activation is required for the neurotoxicity resulting from TNF- α -induced release of MMP-9. In addition, Harnacke et al. [54] noted increases in the amounts of MMP-1 and MMP-9 in TUR human myeloid leukemia cells in which the PARP activity had been stimulated by 12-O-tetradecanoylphorbol-13-acetate. Here, we show that the inhibition of expression of integrin β_1 , MMP-2 and MMP-9 resulting from inhibition of PARP by 5-AIQ may be mediated by decreased NF- κ B activity. Based on our results, we speculate that one mechanism by which PARP induces tumor cell adhesion, migration and invasion is due to increased binding of NF- κ B to DNA, which leads to up-regulation of NF- κ B-dependent genes, including integrin β_1 , MMP-2 and MMP-9. Thus, on the basis of our results, we propose a pathway by which PARP induces adhesion, migration and invasion of CT26 cells, partly through the NF- κ B signaling pathways.

In summary, we have presented experimental evidence which suggests that inhibition of PARP could attenuate the metastatic potential of CT26 colon carcinoma cells. We therefore propose that PARP inhibition could be an effective approach for the inactivation of NF- κ B and its dependent genes, such as integrin β_1 , MMP-9 and MMP-2 expression, resulting in the inhibition of metastases. Further research is needed to prove this hypothesis, particularly in vivo.

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