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Piperlongumine Induces Apoptosis in Colorectal Cancer HCT 116 Cells Independent of Bax, p21 and p53 Status

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Abstract. *Background/Aim: Colorectal cancer is a common type of cancer with reported resistance to treatment, in most cases due to loss of function of apoptotic and cell-cycle proteins. Piperlongumine (PPLGM) is a natural alkaloid isolated from Piper species, with promising anti-cancer properties. This study investigated whether PPLGM is able to induce cell death in colorectal carcinoma HCT 116 cells expressing wild-type or deficient in Bax, p21 or p53. Materials and Methods: PPLGM was extracted from roots of Piper tuberculatum. Cell viability was determined by reduction of 3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assay. Cell death was evaluated by acridine orange/ethidium bromide staining and flow cytometry. Plasmid cleavage activity and circular dichroism DNA interaction were also analyzed. Results: PPLGM induced selective cell death in all cell lines (IC_{50} range from 10.7 to 13.9 μM) with an increase in the number of late apoptotic cells and different profiles in cell-cycle distribution. Plasmid DNA analysis showed that PPLGM does not interact directly with DNA. Conclusion: This paper suggests that PPLGM may be a promising candidate in colorectal cancer therapy.*

According to the last GLOBOCAN publication (2012), colorectal cancer is the third most common malignancy in men and the second in women worldwide. Chemotherapeutic resistance and toxicity found in this type of cancer reinforce the need for alternative therapies.

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Extracts from Piper plants have been used in the treatment of different pathologies for centuries (1). Piperlongumine (PPLGM) is an alkaloid obtained from Piper species with promising anticancer activity. Raj *et al*. in 2011 (2), reported that PPLGM induces cancer-selective cell death by elevating reactive oxygen species (ROS) levels, through inhibition of the antioxidant enzyme glutathione S-transferase. As cancer cells have increased ROS levels compared to normal cells, they were more susceptible to cell death induced by oxidative stress (3, 4). This therapeutic strategy has been extensively studied to selectively kill cancer cells (5, 6).

Cell death induced by PPLGM has been related to apoptotic signals such as increased p53 and PUMA expression, cleaved caspase-3, decreased bcl-2 expression and DNA fragmentation (1, 7). However, some studies show that PPLGM activates autophagy in the presence of apoptotic inhibitors (8, 9). In colon cancer cell lines, PPLGM seems to work, at least in part, through the JNK and MEK/ERK pathways, by activating JNK and ERK, which can induce apoptotic enzymes or phosphorylate transcription factors that regulate the expression of pro-apoptotic genes (10, 11).

Apoptosis is a programmed cell death mechanism that occurs through two major pathways: the extrinsic pathway induced by extracellular stress signals and propagated by specific transmembrane receptors, and the intrinsic pathway, mediated by mitochondrial outer membrane permeabilization (12). p53 is a tumor-suppressor protein that induces cell-cycle arrest, apoptosis or senescence when activated by cellular stress. Hundreds of target genes have been associated to p53, including the pro-apoptotic Bax and the cell cycle regulator p21 (13, 14). Bax protein is a Bcl-2 family member that permeabilizes the mitochondrial outer membrane releasing cytochrome c and activating a caspase cascade. p21 is activated after DNA damage, and temporarily arrests cells at G_1 and G_2 checkpoints from cell cycle, providing time to repair DNA damage, preventing mutations (15). p21 also acts as an apoptosis modulator through apoptosis-inducing protein inhibition, up-regulation of Bax

and activation of tumor necrosis factor family members (16). Abnormalities in apoptotic function contribute to the pathogenesis of colorectal cancer and resistance to chemotherapy and radiotherapy (17). Dysfunctional proteins and loss of function mutations in *p53, p21* and *Bax* have been correlated, *in vitro* and *in vivo*, with multidrug resistance of several cancer cells including colorectal carcinoma cells (18, 19).

In this study, the response of the tumor cell line HCT 116 either wild type or deficient in Bax, p21 or p53 proteins to PPLGM was investigated to evaluate its effect in different cell signaling pathways involved in apoptosis-dependent death.

Materials and Methods

Piperlongumine - extraction and purification. The 8(Z)-N-(12,13,14trimethoxycinnamoyl)-3-pyridin-2-one - PPLGM - was provided by the Chemistry Institute of University of São Paulo (CI-USP). Briefly, PPLGM was extracted from the roots of *Piper tuberculatum* with ethyl acetate and, from the crude extract, obtained directly by methanol crystallization (30% yield). The identification was made by comparison with previously spectral data published (20, 21).

Chemical confirmation – High Resolution Mass Spectrometry. PPLGM was diluted in chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA). The ESI(+)MS and tandem ESI(+)MS/MS (MS-2) were acquired using microTof mass spectrometer (QII-TOF Bruker Scientific, Billerica, MA, USA). For data acquisition and processing, TOF control software (Bruker Scientific) was used.

Cell culture. HEK-293 (human embryonic kidney) cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). HCT 116, HCT 116 Bax –/–, HCT 116 p21 –/– and HCT 116 p53 –/– were gently provided by Annete K. Larsen (Université Pierre et Marie Curie - INSERM, Paris). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), 1% penicillin-streptomycin, and maintained in a humidified atmosphere at 37˚C and 5% CO_2. All cell lines were periodically tested for *Mycoplasma* contamination with DAPI staining.

Cell viability assay. HCT 116 and HEK-293 cells were seeded in 96-well plates (1×10^4 and 8×10^3 cells per well, respectively). After cell attachment, a concentration range of 1-15 μM of PPLGM was added for 24 h. Negative controls were treated with the same amounts of DMSO solvent (for all the assays). After treatment, 1 mg/ml MTT dye in serum-free medium was added for 2 h. MTT solution was removed, the formazan violet product was dissolved in 100 μl of dimethyl sulfoxide (DMSO), and absorption was determined at 570 nm (Spectra Max190, Molecular Devices, Silicon Valey, CA, USA). IC_{50} values were determined as described in literature (22).

Clonogenic assay. HCT 116 cells were seeded in a 6-well plate at a density of 4×10^2 cells/well and treated with 0.5, 1, 1.25, 1.5, 2 μM of PPLGM. After 24 h, cells were washed with PBS, and supplemented medium was added for 10 days. Subsequently, cells were fixed with methanol, cell colonies were stained with 1% crystal violet solution and then counted.

Cell morphology assays by Giemsa and scanning electron microscopy (SEM). HCT 116 cells were seeded in rounded coverslips in 24-well plates (5×10^4 cells per well). After cell attachment, PPLGM treatment was added for 24 h. For Giemsa staining, cells were fixed with methanol, stained with 10% Giemsa and analyzed using light microscopy. For SEM, cells were fixed with 3% glutaraldehyde solution, washed with PBS and dehydrated with ethanol (30% to 100%) for 10 min each. The coverslips were incubated with gold particles and examined in a SHIMADZU Superscan SS-500 scanning electron microscope.

Apoptosis assay by acridine orange/ethidium bromide staining and flow cytometry (cell death and cell cycle). HCT 116 cells were seeded in a 6-well plate (5×10^5 per well) and, after cell attachment, treated with PPLGM for 24 h. Experiments were conducted as previously described in a work from our group (23).

Plasmid cleavage activity assays. The DNA cleavage ability of PPLGM diluted in 100% DMSO was examined in order to establish its effect in the conversion of Plasmid DNA pBSK II supercoiled DNA Stratagene, La Jolla, CA, USA) to the open circular and linear DNA form using agarose gel electrophoresis. Experiments were conducted with 300 ng of pBSK II DNA (~30 μmol l-1 bp) in 10 mmol l-1 Hepes Buffer pH 7.5 treated with PPLGM diluted at 0-30 μmol/l concentrations, in a final concentration of 1% of the solvent at 37˚C, in the absence of light for 16 h. All the assays were performed using control reactions with 1% DMSO. Thereafter, each reaction was quenched by adding 4 μl of a loading buffer solution and subjected to electrophoresis on a 1.0% agarose gel containing 0.3 μg/ml EB in 0.5× TBE. The resulting gels were visualized and digitized using a DigiDoc-It gel documentation system (UVP) (KODAK, Rochester, NY, USA). The proportion of plasmid DNA in each band was quantified using Kodak Molecular Imaging Software 5.0 (Carestream Health, Rochester, NY, USA). The quantification of supercoiled DNA was corrected by a factor of 1.47, since the ability of EB to intercalate into this DNA topoisomeric form is decreased relative to open circular and linear DNA (24).

Circular dichroism (CD) DNA interaction assay. CD is a method able to detect alterations in optical activity of chiral molecules using their interaction with circularly polarized electromagnetic rays. The B-form conformation of Calf Thymus DNA (CT-DNA) shows two CD bands in the UV region, a positive band at 278 nm due to base stacking and a negative band at 246 nm due to polynucleotide helicity. The experiments were performed as described in the literature with small modifications (25). One sample of 2000 μM CT-DNA in 10 mM of HEPES buffer was titrated with PPLGM diluted in 100% DMSO in concentrations ranging from 9.95 to 95.24 μM and 0 (negative control). The screenings were realized in the range of 220 to 600 nm at 37˚C immediately after the addition of the complexes.

Statistical analysis. The results were expressed as mean±standard deviation obtained from three independent experiments performed in triplicate, used to assess normal distribution data. Statistical

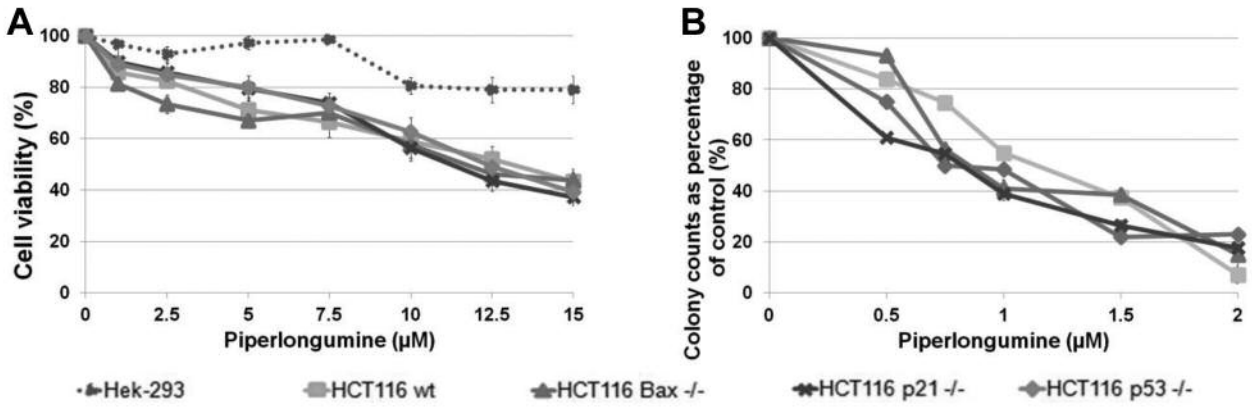

Figure 1. *Cytotoxic activity from PPLGM. (A) Cell viability after PPLGM exposure in wild-type and deficient HCT 116 cell lines and non-tumor HEK-293 cell line. Cells were treated with different concentrations of PPLGM for 24 h and viability was measured by the MTT assay. Mean±SD are shown from 3 independent experiments. (B) Effects of PPLGM on colony formation in wild-type and mutant HCT 116 cells (colonies counted and plotted as percentage of the control number of colonies).*

significance was evaluated using *t*-test and one-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey test) to assess statistical differences in normal distribution. Statistical significance was accepted at a level of $p<0.05$. All statistical analyses were performed using the SPSS statistical software (version 23.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results

PPLGM is an alkaloid, which was first isolated and characterized by Navickiene *et al*. (2000). Chemical structure confirmation was performed by HRMS were was possible to observe the molecular ion $(M+H)^+$ m/z 318.1356 (data not shown).

To evaluate cytotoxic activity of PPLGM, the MTT and the clonogenic assay were performed. The viability of HCT 116 and HEK-293 cell lines after treatment with PPLGM is represented in Figure 1A. MTT assay showed that PPLGM induces cell death in HCT 116 cells independent from Bax, p21 and p53 status, but reduced cytotoxicity against the non-tumor cell line HEK-293 at concentration ≥10 μM. PPLGM also reduced significantly the clonogenic capacity of HCT 116 cells after 24 h treatment (Figure 1B). The IC_{50} values from both assays are reported in Table I. For the MTT assay, the resistance index (R.I.) between the IC_{50} from each cell line was compared with HCT 116 wild-type, showing a very similar response pattern.

Cell lines exhibited typical apoptotic characteristics after PPLGM treatment (Figure 2). Untreated HCT 116 cells exhibited normal features with typical adherent morphology. After 24 h of exposure to PPLGM, cells developed a rounded morphology, with membrane shrinkage, nuclear condensation and loss of adherence. Scanning electron microscopy highlighted the presence of apoptotic bodies (E).

Table I. *Piperlongumine IC_{50} values obtained from MTT and clonogenic assays in different HCT 116 cell lines and HEK-293.*

Cell line	MTT assay IC_{50} (μM)	R.I[a]	Clonogenic assay IC_{50} (μM)
HEK - 293	N.A.	-	N.A.
HCT 116 wt	12.8±1.0	1.0	1.22
HCT 116 Bax –/–	13.9±1.3	1.1	0.90
HCT 116 p21 –/–	12.2±0.5	0.95	1.03
HCT 116 p53 –/–	10.7±0.9	0.85	0.95

[a]Resistance index (R.I.) for MTT assay was calculated as the ratio between the IC_{50} values obtained for each cell line and HCT 116 wild-type. N.A: Not available.

The AO/BE staining revealed the presence of membrane blebbing and apoptotic bodies in HCT 116 cells treated with PPLGM (G-I).

Control and PPLGM-treated HCT 116 cells were evaluated by flow cytometry subsequent to Annexin V and PI staining. After 24 h treatment, there was an increase in the percentage of late apoptotic/necrotic cells in PPLGM exposed cells (data not shown). At the higher tested concentration, late apoptosis and/or necrosis events ranged from 16.5-37.9%. To determine whether the cytotoxic effect of PPLGM on colorectal cells was associated with induction of cell cycle arrest, cell distribution in cycle phases was analyzed by flow cytometry. Piperlongumine induced G_2/M cell-cycle arrest in Bax, p21 and p53 deficient cells (data not shown).

To evaluate PPLGM genotoxicity, *in vitro* methods were performed using plasmid DNA and CT-DNA. Cleavage activity assay showed that in the presence of DMSO PPPLGM presented no significant cleavage activity on plasmid DNA,

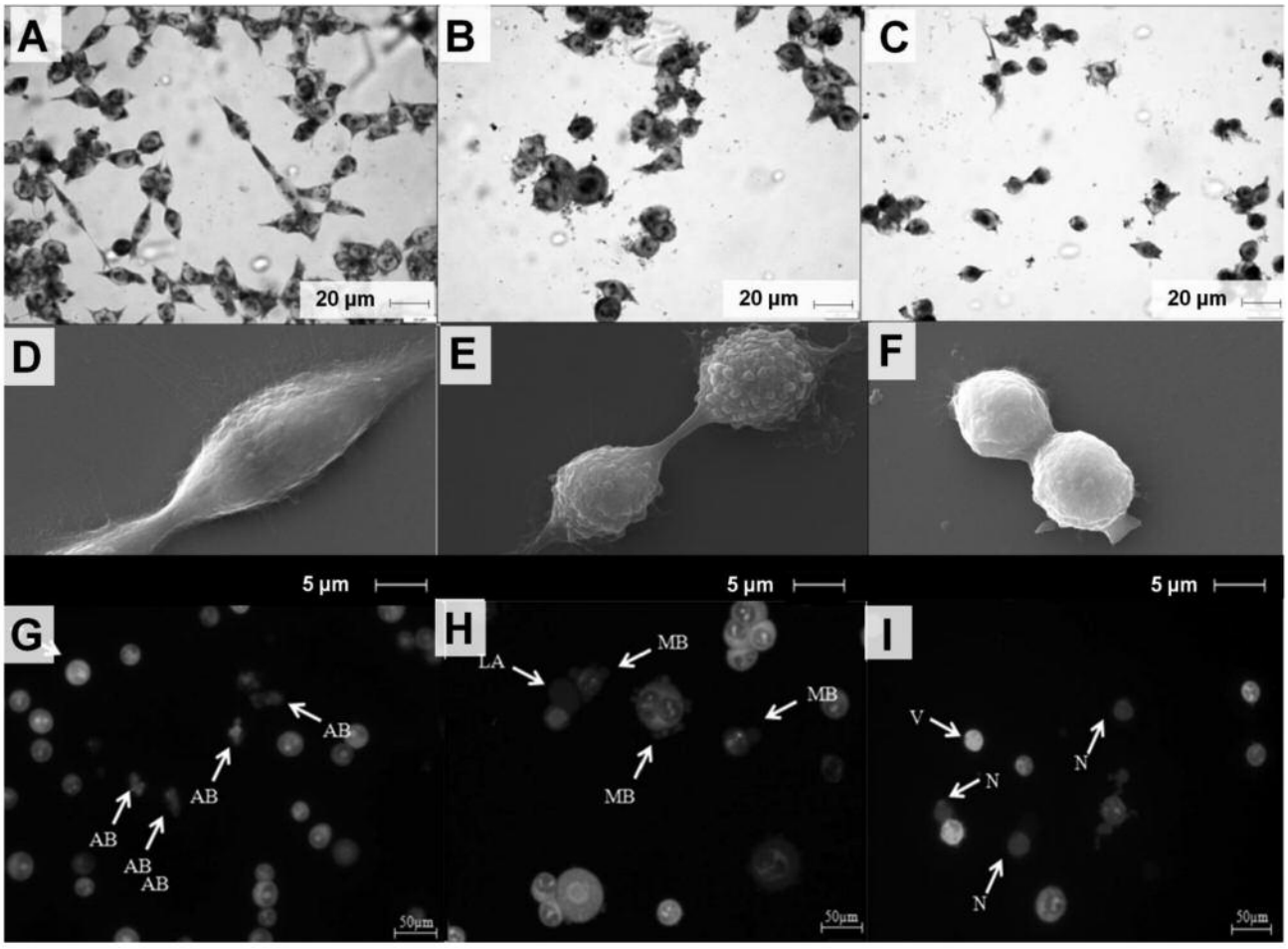

Figure 2. *(A-I) Morphological changes in HCT 116 wild-type after 24 h exposure to 6 μM (B, E) and 12 μM (C, F) PPLGM treatment. Cells were fixed and stained with Giemsa (A-C) and analyzed by scanning electron microscopy (D-F). Treated cells showed rounded morphology, membrane shrinkage, nuclear condensation and apoptotic bodies (E). (G-I) Typical morphological changes in HCT 116 cells after PPLGM treatment stained with AO/EB. The images were taken using a fluorescence microscope at ×400 magnification. V: Viable cells; AB: apoptotic bodies; MB: membrane blebbing; LA: late apoptosis; N: necrotic cell.*

with no significant increase of the open circular form of DNA (data not shown). In circular dichroism assay, PPLGM was not able to induce differences in circularly polarized electromagnetic rays reading (data not shown).

Discussion

The anti-cancer effect of PPLGM has been previously reported (1, 2, 7-11, 26, 27). PPLGM was screened and showed no cytotoxicity in six non-tumor cell types, but cytotoxic effects were observed with an average IC_{50} of 7 μM in thirteen cancer cell lines (2). Here, the effects of PPLGM on HCT 116 colon cancer cell lines (wild-type, Bax –/–, p21 –/– and p53 –/–) and non-tumor HEK-293 cell viability were investigated and demonstrated a selective cell death pattern in cancer cells, with an IC_{50} range from 10.7 μM to 13.9 μM. These results are similar to Randhawa *et al*. (10) results, where PPLGM was shown to be toxic toward HT-29 and HCT 116 colon cancer cells, with an IC_{50} of 6.4 μM and 10.1 μM, respectively.

Several studies have shown that PPLGM induces cell death through different pathways in multiple types of cancer cells (2, 8-10). In this study, apoptotic events in HCT 116 wt, Bax –/–, p21 –/– and p53 –/– HCT 116 cells exposed to PPLGM were observed and evidenced by chromatin condensation, membrane blebbing and presence of apoptotic bodies. Morphological evaluation showed that PPLGM treatment leads to membrane shrinkage, with treated cells showing rounded shapes, distinct from untreated ones. The Annexin-V/PI assay showed that HCT 116 proficient and deficient cells were induced to apoptotic and necrotic cell

death, in a PPLGM concentration-dependent manner. Previous studies (10, 11) also found that PPLGM treatment led to apoptosis in HT-29 and HCT 116 colorectal cancer cell lines, *via* induction of ERK and JNK signaling pathways, respectively. HCT 116 wild-type cells showed no alterations in cell-cycle distribution. This result corroborates with Li *et al*., 2015 (11), who found no effect of PPLGM on the cell cycle distribution of HCT116 wild type cells, suggesting that the loss of viability in these cells may be attributable to cell death.

Interestingly, HCT 116 Bax –/–, HCT 116 p21 –/– and HCT 116 p53 –/– showed a significant increase in G_2/M phase after PPLGM exposure. Cell cycle arrest at G_2/M prevents cells with damaged DNA from entering mitosis, providing an opportunity for repair and stopping the proliferation of damaged cells (26). PPLGM also induced G_2/M arrest in V79, gastric cancer and cholangiocarcinoma cells, similarly to topoisomerase I (campthotecin) and II (etoposide) (27, 28). In the present study, telomerase activity was not evaluated.

Colorectal cancer is a very common malignancy with poor prognosis, especially when diagnosed in late stages. The currently used chemotherapeutic combination agents are frequently associated with acquiring resistance, which can be related to multifactorial mechanisms, including deregulation of cell cycle and cell death pathways. Previous studies with HCT 116 Bax –/– cells demonstrated that deficient cells provided partial protection against camptothecin and cisplatin and resistance to indomethacin, sulindac acid and 5-FU (29-33). In our study, PPLGM induced apoptosis in both wild-type and Bax –/– HCT 116 cells, with no significant difference in the IC_50, which led us to believe that PPLGM can be a promising anticancer target against mutated tumor cells.

The p53 protein regulates the response to genotoxic stress by activation or repression of genes encoding proteins involved in cell cycle control, DNA repair and apoptosis (34). Loss of p53 function has been correlated with multidrug resistance in many tumor types (35). p21 protein can be activated by both p53 dependent and p53 independent mechanisms. p21 has a significant role in modulating DNA repair processes, inhibiting cell cycle progression to allow DNA repair while inhibiting apoptosis. p21 synergizes with tumor suppressors and antagonizes with oncogenes to protect against cancer development. Many human cancers such as colorectal and cervical are associated with reduced p21 expression (16).

Previous studies reported that PPLGM increases p53, p21 and Bax expression in colorectal carcinoma, myeloid leukemia and oral squamous carcinoma respectively (2, 36, 37). In our study, PPLGM treatment induces cell death independent from Bax, p21 and p53 status, which corroborates with the fact that, although PPLGM induces the expression of these proteins, they are not fully requested in the apoptotic mechanism induced by PPLGM.

Increased ROS levels can result in many types of DNA damage, including single and double-strand breaks, base modifications, deoxyribose modification and DNA cross linking. In plasmidial analysis, a DMSO solution with PPLGM showed no genotoxic effects. As DMSO is considered a ROS scavenger, this result indicates that PPLGM effects on DNA are probably dependent on the production of ROS. This result corroborates with Dhillon's *et al*. results (38), where PPLGM-induced DNA damage in pancreatic cancer cells was reversed by treating cells with an exogenous antioxidant.

Conclusion

PPLGM is a potent inhibitor of proliferation of HCT 116 cells that induces apoptosis independent of Bax, p21 and p53 status, which supports the fact that PPLGM can be a potential anti-cancer agent.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

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