PTC209, a Specific Inhibitor of BMI1, Promotes Cell Cycle Arrest and Apoptosis in Cervical Cancer Cell Lines

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Abstract. *Background/Aim:* Aberrant expression of the *BMI1* oncogene has been prevalently found in a variety of human cancers, including cervical cancer. Recent studies have shown that *PTC209*, a specific *BMI1* inhibitor, exhibits high potency in inhibiting the growth of colon, breast, oral cancer cells and cancer-initiating cells, indicative of its chemotherapeutic potential. In the current study, we evaluated the inhibitory abilities of *PTC209* in cervical cancer cells. *Materials and Methods:* Three cervical cell lines, C33A, HeLa, and SiHa were treated with *PTC209*. The impacts of *PTC209* on *BMI1* were investigated using quantitative reverse-transcription PCR assay (qRT-PCR) and western blotting; changes in cell viability, cell cycle distribution, and apoptosis were assessed using cell viability testing, colony formation assay and flow cytometry analyses, respectively. *Results:* *PTC209* exhibited considerably high short-term and long-term cytotoxicities in all tested cervical cancer cell lines regardless of their HPV infection status, TP53 and pRb statuses. *PTC209* significantly downregulated the expression of *BMI1* in cervical cancer cell lines, and such downregulation led to G0/G1 arrest ($p < 0.05$). Moreover, *PTC209* drove more cells into apoptosis ($p < 0.05$). *Conclusion:* *PTC209* (*BMI1*-targeting agents, in general) represents a novel chemotherapeutic agent with potential in cervical cancer therapy.

Cervical cancer is the fourth most common malignancy affecting women worldwide with an ascending incidence and youthful trends in developing countries, and about 250,000 deaths have been estimated annually (1, 2). In the United

States, more than 13,000 women will be newly diagnosed with cervical cancer and about 4,200 women will die from this malignancy in 2019 (1). Since human papillomavirus (HPV) is found in about 99% of cervical cancers, the etiology of cervical cancer has been widely recognized as a result of HPV infection (3, 4). However, it is known that HPV infection alone is not sufficient to generate a fully malignant phenotype (5). The molecular mechanisms underlying the malignant transformation and progression of cervical cancer remain poorly understood (6-8).

Recent studies have shown that polycomb complex protein B-lymphoma Mo-MLV insertion region 1 (*BMI1*) is associated with the development and progression of a variety of human cancers, including colon, oral, breast, ovarian, endometrial, biliary tract, liver, gastric, prostate, pancreatic, and lung and cervical cancer (9-14). *BMI1* is a key protein partner in polycomb repressive complex 1 (PRC1) that represses gene transcription by mono-ubiquitylation of histone 2A at Lys 119 (H2AK119ub) (11, 12, 15, 16), and it impacts gene expression pattern involved in cell proliferation, growth, DNA repair, apoptosis, and senescence (9, 15, 16). Aberrant expression of *BMI1* at the mRNA or protein level has been prevalently found in human cancers, and correlates with advanced disease stages, aggressive clinicopathological behavior, poor prognosis, and resistance to radiation and chemotherapy (5, 9, 14, 16-22). Therefore, *BMI1* is emerging as a potential chemotherapeutic target in cancer therapy. A low-molecular-weight compound, *PTC209* (Figure 1A) has been found to be able to downregulate the expression of *BMI1* in a number of breast, colon, prostate, and biliary tract cancer cells and cancer-initiating cells, resulting in inhibition of cell proliferation, induction of apoptosis and/or senescence, increased susceptibility to cytotoxic agents and radiation therapy, inhibition of epithelial mesenchymal transition (EMT) and cancer stemness (15, 23-31). In regard to the fact that aberrant *BMI1* expression is prevalent in cervical cancer patients, we

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Key Words: *PTC209*, *BMI1*, inhibition, cervical cancer, cancer stem cell.

hypothesized that PTC209 would exhibit inhibitory potency in cervical cancer cells. In this study, we evaluated the impact of PTC209 on cell proliferation and apoptosis in cervical cancer cells. Our results showed that PTC209 exhibited considerable cytotoxicity in three tested cervical cancer cell lines, C33A, HeLa, and SiHa. PTC209 significantly downregulated *BM11* expression in these cells, resulting in G₀/G₁ arrest and apoptosis. Our results support further studies on PTC209 as a novel chemotherapeutic agent in cervical cancer therapy.

Materials and Methods

Cell lines and reagents. Three cervical cancer cell lines, C33A, HeLa, and SiHa were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The characteristics of these cervical cell lines are summarized in Table I. All cell lines were maintained in advanced Dulbecco modified Eagle medium (DMEM) medium (Life Technologies Corporate, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Life Technologies), 1% glutamine (Life Technologies), 1% penicillin/streptomycin (Life Technologies) at 37°C, in a humidified atmosphere with 5% CO₂. These cell lines were regularly authenticated using short tandem repeat polymorphism (STRP) analysis as recommended by ATCC, and they were mycoplasma free. Cells were grown up to passage 20. PTC209 (CAS 315704-66-6; Figure 1A) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Gene expression assay. Cells were seeded at 1×10⁶ cells/T25 flask (Life technologies) and incubated in DMEM-5% FBS media overnight. Cells were then incubated with media containing PTC209 at indicated concentrations (0 μM, 1 μM, 2 μM, 5 μM, and 10 μM;) at 37°C and 5% CO₂ for another 24 h. Cells were harvested by trypsin and centrifugation and total RNA was purified using a RNeasy Purification kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized using a High Capacity cDNA Reverse Transcription kit (Life Technologies). The expression levels of target genes were quantitatively assessed using Taqman® gene expression assays (Life Technologies) using the following inventoried primer/probes: Hs00409825_g1 for *BM11*, and Hs99999909_m1 for human hypoxanthine phosphoribosyltransferase (*HPRT1*). Of note, *HPRT1* was used as an endogenous reference for normalized gene expression. PTC209 treatment experiments were performed in triplicate. For each cDNA sample, target genes were amplified separately, and expression quantitation assays were performed in triplicate. The relative gene expression level (REL) of *BM11* (in comparison with *HPRT1*) was determined using a comparative C_q method in which REL was defined as 2^{-δC_q} (32).

Western blot. Cell pellets were washed three times in ice-cold PBS (phosphate saline, pH 7.4) and then lysed with RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, and 1 μg/ml of leupeptin; Cell Signaling Technology, Beverly, MA, USA] on ice for 20 min. Subsequently, cell lysates were clarified by centrifugation at 4°C, 13,000 rpm for 5 min and the concentration of total proteins in the supernatants was determined using the BCA assay (Thermo Fisher Scientific). The samples were subjected to SDS-PAGE (10%

Figure 1. PTC209 downregulated the expression of *BM11* in cervical cancer cells. A) The chemical structure of PTC209. B) The expression of *BM11* mRNA in cervical cancer cells decreased in the presence of PTC209. Pre-validated qRT-PCR-based assays were used to evaluate the mRNA expression levels of target genes. While *HPRT1* was used as an endogenous reference for gene expression, cells without PTC209 treatment were used as controls. The relative expression level of *BM11* mRNA was defined using the 2^{-δC_q} method. PTC209 treatment experiments were performed in triplicate. Each expression quantitation assay was repeated at least three times. Data are presented as mean±standard deviation. For each cell line, group-wise comparisons were between the groups with PTC209 at indicated concentrations (1 μM and 2 μM) and the group without PTC209 treatment (0 μM). **p*<0.05; ***p*<0.01; ****p*<0.001. C) Western blot analysis of *BM11* protein. Samples containing 50 μg of total soluble proteins per lane were subjected to SDS-PAGE for separation, and transferred to nitrocellulose membrane followed by immunoblotting using following antibodies: mouse anti-*BM11* monoclonal antibody (sc-390443, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-β actin monoclonal antibody (sc-56459, Santa Cruz Biotechnology), rabbit anti-mouse IgG (H+L) secondary antibody conjugated with horse radish peroxidase (HRP) (A16160, Thermo Fisher Scientific). β-Actin was used as an endogenous control.

Table I. Characteristics of cervical cancer cell lines*.

Cell	Morphology	Origin	Molecular characteristics
C33A	Epithelial	Cervical adenocarcinoma	HPV16-negative, HPV18-negative; p53+, highly expressed, R273C; pRb+, abnormal in size.
HeLa	Epithelial	Cervical retinoblastoma	HPV16-negative, HPV18-positive; p53+, lowly expressed; pRb+, normally expressed.
SiHa	Epithelial	Cervical squamous cell carcinoma	HPV16-positive, HPV18-negative; p53+; pRb+.

*Information from American Type Culture Collection (www.atcc.org)

or 15%) at 50 µg of total proteins per lane and then transferred to a nitrocellulose membrane and immunoblotted with the following antibodies: mouse anti-BMI1 monoclonal antibody (sc-390443, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-β actin monoclonal antibody (sc-56459, Santa Cruz Biotechnology), rabbit anti-mouse IgG (H+L) secondary antibody conjugated with horse radish peroxidase (HRP) (A16160, Thermo Fisher Scientific). The concentration of each antibody was used as suggested by the suppliers. Immunodetection was performed using the enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific) (33).

Cell viability assay. Cells were seeded at 2000 cells/well in 100 µl of advanced DMEM- 5% FBS media and incubated at 37°C and 5% CO₂ overnight. Subsequently, cells were incubated with media containing various concentrations of PTC209 for another 24 h. Cell viability was assayed using WST-1 Cell proliferation Assay kit (Roche, Indianapolis, IN, USA) following the manufacturer's directions. Assays were performed in triplicate at least twice. Absolute IC₅₀ values (the concentration of cisplatin required to inhibit 50% of the cell viability) were determined using Kaleidagraph software (Synergy Software, Reading, PA, USA) as previously described (34).

Colony formation assay. Cells were seeded in 12-well plates at 500-1,000 cells/well and grown at 37°C and 5% CO₂ for 24 h. Cells were then treated with PTC209 at indicated concentrations for 7-10 days. Growth media were changed with or without drugs every other day. When the colony could be seen by naked eyes, the culture was terminated. After removal of the media, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 m, and stained with a 0.5% crystal violet solution for 2-4 h. Plates were washed with tap water to excessive staining solution. The colonies were counted and photographed (35, 36). Three duplicates were set up in each group, and each reaction was run in triplicate. The colony formation ability was defined as the number of colonies at the indicated concentration of PTC209/the number of colonies without PTC209×100%.

Flow cytometry for cell cycle distribution. After PTC209 treatment for 24 h, cells (1×10⁶) were collected by trypsin and centrifugation and washed with pre-cold PBS. Subsequently, cells were fixed in 70% ethanol at 4°C for 12 h, followed by incubation in 50 µg/ml RNase A solution (BD Biosciences, San Jose, CA, USA) at 37°C for 30 min. Samples were incubated with 10 g/ml propidium iodide (BD Biosciences) in the dark for 15 m, and then analyzed by Attune NxT flow cytometer (Thermo Fisher Scientific, Rockford, IL, USA) (35). Each treatment was conducted in triplicate, and each sample was analyzed at least twice.

Flow cytometry for apoptosis. After treatment with PTC209 at indicated concentrations for 24 h, cells (1×10⁶) were collected and stained with FITC Annexin-V/PI Apoptosis Detection kit I (BD Biosciences) following the manufacturer's instructions. Cells were subjected to Attune NxT flow cytometer (Thermo Fisher Scientific) to measure apoptosis (37). Each treatment was conducted in triplicate, and each sample was analyzed at least twice.

Statistical analyses. R3.4 (R: The R Project for Statistical Computing, <https://www.r-project.org>) was used in this study. Measurement data were presented as mean±standard deviation (SD). Group-wise comparisons were analyzed using Student's *t*-tests, and no multiple comparison adjustment was applied. Of note, gene expression data were subjected to log transformation to fulfill the normality requirement prior to group-wise comparison. All tests were two-sided and the significance level was preset at $\alpha=0.05$. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Results

PTC209 potently downregulated the expression of BMI1 in cervical cancer cells. It has been reported that PTC209 specifically decreases BMI1 at the transcription and/or protein level in a variety of cancer cell lines and patient-derived cancer cells, whereas the underlying molecular mechanisms remain to be further elucidated. In the present study, we first evaluated the impacts of PTC209 on BMI1 in three cervical cancer cell lines, C33A, HeLa, and SiHa. These three cell lines were selected for the following reasons: while HPV infection is found in almost all cervical cancer specimens (>99%), HPV16 and HPV18 are the two major high-risk HPV types that may cause cervical cell abnormalities or cancer, and more than 70% of cervical cancer cases can be ascribed to HPV16 and HPV18 (3, 4). In the meanwhile, abnormalities in tumor suppressive *TP53* and *pRb* genes are the most prominent genetic alterations found in human cancers (32, 38). As summarized in Table I, C33A, HeLa, and SiHa have different molecular characteristics, which jointly may represent the wide scope of cervical cancer cells.

In the absence of PTC209, the baseline expression levels of *BMI1* mRNA varied considerably among these three cells (Figure 1B). While HeLa and SiHa had comparable baseline

expression levels of *BMI1* mRNA ($p>0.05$), the baseline expression level of *BMI1* mRNA in C33A appeared to be relatively high (p -values <0.05 in comparison with HeLa or SiHa). Interestingly, PTC209 downregulated the expression of *BMI1* mRNA in all three cell lines. After a 24-h incubation, 1 μ M PTC209 reduced the expression of *BMI1* mRNA by 5-10-fold in these cell lines (all p -values <0.01). Two μ M PTC209 brought about more than 100-fold decrease in *BMI1* mRNA expression (all p -values <0.001), whereas the expression of *BMI1* mRNA was undetectable in these cells with high concentrations of PTC209 (5 μ M and 10 μ M, data not shown). These observations were further supported by immunoblotting analyses of BMI1 protein in these cells. As shown in Figure 1C, the baseline levels of BMI1 protein in these three cell lines appeared to be different, and 1 μ M PTC209 decreased the level of BMI1 protein in all three cell lines. Of note, BMI1 protein was undetectable in all three cell lines with higher concentrations of PTC209 (2 μ M, 5 μ M and 10 μ M, data not shown). With regard to the different characteristics of these three cell lines (Table I), these results indicate that PTC209 is a potent inhibitor that downregulates BMI1 in cervical cancer cells regardless of HPV infection and genetic statuses of *TP53* and *pRb*.

PTC209 inhibited the growth of cervical cancer cells. We then evaluated the cytotoxicity of PTC209 in these cervical cancer cells. Results from cell viability assays demonstrated that PTC209 exhibited considerable inhibitory activity in these cells after 24 h treatment. The IC_{50} values of PTC209, *i.e.* the concentrations required to inhibit 50% of cell viability, were 12.4 ± 3.0 μ M, 4.3 ± 1.8 μ M, and 21.6 ± 4.2 μ M in C33A, HeLa, and SiHa, respectively (Figure 2A). HeLa appeared to be more sensitive to short term (24 h) treatment with PTC209 as evidenced by an IC_{50} value lower than that of C33A (by 3-fold) or SiHa (by 5-fold). Further, colony formation assays showed that PTC209 exhibited very high long-term (up to 7 days) cytotoxicity as evidenced by the fact that 2 μ M PTC209 inhibited the colony formation abilities of these cells by more than 50% (Figure 2B). While only remnant colony formation abilities were observed in C33A and SiHa in the presence of 5 μ M PTC209, HeLa retained about 35% and 20% of colony formation ability in the presence of 5 μ M and 10 μ M PTC209, respectively, implying that HeLa might be less sensitive to PTC209 after long term treatment than C33A or SiHa. Apparently, this observation is different to a certain extent from our previous results showing that HeLa was more sensitive to PTC209 after short term treatment. The mechanisms underlying such “disparity” between short- and long-term cytotoxicities of PTC209 in HeLa remain to be further explored.

PTC209 induced G_0/G_1 arrest and apoptosis in cervical cancer cells. Subsequently, we investigated the potential impact(s) of

PTC209 in the cell cycle progression of these cells. Flow cytometry analyses showed that with 24 h incubation, there was a statistically significant increase in the number of cells at the G_0/G_1 phase upon PTC209 treatment (5 μ M and 10 μ M) compared to the untreated cells (all p -values <0.05) (Figure 3). The proportion of cells in the G_0/G_1 phase increased from 57.9% (0 μ M) to 64.7% (5 μ M) and 64.1% (10 μ M) in C33A, from 53.2% (0 μ M) to 60.4% (5 μ M) and 63.9% (10 μ M) in HeLa, from 61.3% to 68.3%, and 67.2% in SiHa. Accordingly, numbers of cells in the S phase and/or the G_2/M phase decreased upon PTC209 treatment.

Lastly, we examined the apoptotic effect of PTC209 in these cells using annexin V and propidium (PI) staining followed by flow cytometry analyses. Our results demonstrated that after treatment for 24 h, PTC209 significantly increased total apoptosis (annexin V⁺/PI⁻ and annexin V⁺/PI⁺) in these tested cells in a concentration-dependent manner (Figure 4). After 24 h treatment with PTC209, about 6.6% (5 μ M) and 10.5% (10 μ M) of C33A cells underwent apoptosis compared to 3.5% in untreated C33A cells (0 μ M). Similarly, PTC209 treatment increased the numbers of HeLa apoptotic cells from 10.3% (0 μ M) to 19.2% (5 μ M) and 34.1% (10 μ M). For SiHa cells, about 4.3% cells underwent apoptosis without PTC209 treatment, and such percentages increased to 8.1% and 15.4% upon treatments with 5 μ M and 10 μ M PTC209, respectively. Interestingly, in 10 μ M PTC209-treated HeLa cells, about 30% cells were in the early apoptotic stage (annexin V⁺/PI⁻) and 4.1% cells were in the late apoptotic stage (annexin V⁺/PI⁺). In comparison, about 5.5% and 10.0% of 10 μ M PTC209-treated SiHa cells were in early apoptosis and late apoptosis, respectively. As for 10 μ M PTC209-treated C33A cells, the proportions of cells in early apoptosis and late apoptosis were about 5.8% and 4.7%, respectively.

Discussion

PcG-mediated transcriptional repression provides universal mechanisms to modulate genes involved in embryonic development, cell proliferation and apoptosis, maintenance of cellular identity, and aberrant functioning of PcG complexes has been shown to correlate with diverse human cancers (9, 15). PcG proteins assemble into multi-component complexes that remodel local chromatin structure both directly and through the establishment and removal of post-translational modifications of histone proteins, thus influencing the accessibility of target genes to transcription factors. One major PcG complex, PRC1, specifically monoubiquitylates histone Lys 119 through its ubiquitin E3 ligase activity, and consequently silences a number of target genes, one of which is the *INK4a-ARF* gene (11, 16). The *INK4a-ARF* gene encodes p16INK4A and p14ARF proteins, which negatively regulate the prominent CDK4-pRb pathway and the MDM2-p53 pathways, respectively (38). Hence, as an essential

Figure 2. PTC209 inhibited the growth of cervical cancer cells. A) Short-term cytotoxicity. Cervical cancer cells were incubated with various concentrations of PTC209 for 24 h. Cell viabilities were then evaluated using WST-1 cell proliferation Assay kit following the manufacturer's instruction, and IC_{50} values, the concentrations of cisplatin required to inhibit 50% of the cell viability, were calculated using a 4-parameter non-linear regression approach. Experiments were conducted at least in triplicate. B) Long-term cytotoxicity. Cervical cancer cells were plated in 12-well pates (about 500 cells/well) and incubated in regular media for 24 h followed by media containing indicated concentrations of PTC209 (0, 1, 2, 5, and 10 μM) for another 7 days. After fixation with 4% paraformaldehyde, cells were visualized under a microscope. Three duplicates were set up in each group, and each reaction was run in triplicate. The colony formation ability was defined as the number of colonies at the indicated concentration of PTC209/the number of colonies without PTC209 $\times 100\%$.

component of PRC1, BMI1 is involved in the regulation of pRb-mediated cell cycle control and p53-mediated apoptosis as well as other related cellular process (9, 38, 39). It is no wonder that BMI1 deregulation (such as aberrant expression of *BMI1*) has been clearly implicated in the tumorigenesis and development of many human cancers (9-14). Here, we

reported that PTC209-mediated downregulation of BMI1 in cervical cancer cells induced G_0/G_1 arrest and apoptosis, thus exhibiting considerably high short-term and long-term cytotoxicities. Our results are consistent with previous studies showing that PTC209 treatment led to cell cycle arrest and apoptosis in many cancer cells *in vitro* and *in vivo* (23-31),

Figure 3. PTC209 induced G₀/G₁ arrest in the cervical cancer cell lines. C33A (A), HeLa (B), and SiHa (C). Cells (about 1×10^6) were treated with PTC209 at indicated concentrations (0, 5, and 10 μ M) for 24 h, and then fixed in 70% ethanol at 4°C overnight. After incubation with RNase A and propidium iodide, the cells were analyzed using the Attune NxT flow cytometer (Thermo Fisher Scientific). Each treatment was conducted in triplicate, and each sample was analyzed at least twice. Data were presented as mean $\pm$ SD. For each cell line, the percentage of cells at a specific stage after treatment with PTC209 at indicated concentrations (5 μ M and 10 μ M) was compared with that without PTC209 treatment (0 μ M) using two-sided Student's *t*-test. **p*<0.05; ***p*<0.01; ****p*<0.001.

Figure 4. PTC209 promoted apoptosis in the cervical cancer cell lines. C33A (A), HeLa (B), and SiHa (C). Cells (about 1×10^6) were treated with PTC209 at indicated concentrations (0, 5, and 10 μM) for 24 h, and stained with FITC Annexin-V/PI Apoptosis Detection kit I (BD Biosciences) following the manufacturer's instructions. Cells were analysed using the Attune NxT flow cytometer (Thermo Fisher Scientific) to measure apoptosis. Each treatment was conducted in triplicate, and each sample was analyzed at least twice. Data are presented as mean $\pm$ SD. For each cell line, the percentage of cells at a specific stage after treatment with PTC209 at indicated concentrations (5 μM and 10 μM) was compared with that without PTC209 treatment (0 μM) using two-sided Student's *t* test. Early apoptosis, annexin V⁺/PI⁻; late apoptosis, annexin V⁺/PI⁺; combined, annexin V⁺/PI⁺ and annexin V⁻/PI⁺. **p*<0.05; ***p*<0.01; ****p*<0.001.

indicative of the potential of PTC209 as a novel chemotherapeutic agent in cancer therapy. This notion is further supported by recent studies (17, 18) demonstrating that upregulation of *BMI1* is associated with chemoresistance and tumor recurrence in several cancer types, and PTC209-mediated inhibition of *BMI1* in primary or metastatic tumors may improve response to chemotherapeutic agents, such as carboplatin, doxo-rubicin, and castration.

Recent studies have also identified *BMI1* as a key factor in the maintenance and/or self-renewal of many cancer stem cell types, including embryonic, neural, hematopoietic and prostate (15, 27-29, 40, 41). While *BMI1* overexpression correlates with other stemness-related genes, such as *SOX2*, *KLF4*, *NANOG*, and *gankyrin*, in cervical, breast and head and neck cancers (8, 10, 40, 41), it has been demonstrated that the function of cancer-initiating cells (CICs) in colorectal cancer, breast cancer, and glioblastoma is dependent on *BMI1* and PTC209-mediated downregulation of *BMI1* inhibits these CICs to self-renew, resulting in the abrogation of their tumorigenic potential (15, 28, 29, 40).

Taken together, results from our laboratory and other groups reveal that *BMI1* represents a novel target in both anti-cancer and anti-CSC therapies and it is valuable to further investigate the potentials of PTC209 as a single therapeutic agent or in combination with other chemotherapeutic agents in cervical cancer therapy (30, 42).

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

JL and MP designed the experiments, analysed the data, and wrote the manuscript. JL and ZV performed the experiments. All the Authors read and approved the final manuscript.

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Received December 4, 2019

Revised December 10, 2019

Accepted December 12, 2019