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RESEARCH ARTICLE

Berberine/Cisplatin based radiosensitization in HPV positive cervical cancer cell line HeLa is mediated through activation of mitochondrial pathway of cell death

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Abstract

This study compared the existing therapy regime with a Berberine/Cisplatin based radiotherapy in cervical cancer *in vitro*. Treatment of cervical cancer HeLa cells with berberine/cisplatin combination followed by treatment by ionizing radiation (IR) resulted in increased apoptosis in comparison to cisplatin based radiotherapy. The combination therapy resulted in upregulation of pro apoptotic proteins like Bax, p73 and down regulation of anti apoptotic BclXl, inflammatory Cox-2; Cyclin D1 accompanied by increase in activity of Caspase-3 and -9. Reduction in telomerase activity was also seen in the HPV positive cells. Thus this combination offers potential for treatment of cervical cancer.

Keywords: Berberine, cisplatin, cervical cancer, HeLa, radiotherapy, apoptotic proteins, telomerase.

Introduction

Cervical cancer remains one of the major cancers among women worldwide with a high rate of mortality (Castellsague, 2008). Radiotherapy in combination with cisplatin remains the treatment of choice in majority of cases in which the cancer is locally advanced. Currently the chemotherapeutic drugs cisplatin and 5-Fluorouracil are used as radiosensitizers along with ionizing radiation (IR) for the treatment of cervical cancer (Candelaria *et al.*, 2006). Nevertheless, therapeutic results are far from optimal, so new and safer therapeutic combinations need to be investigated which specifically target cervical cancer cells with minimal toxicity to normal tissue. Although chemo radiotherapy is more effective as compared to radiotherapy alone, it is associated with dose limiting toxicities like gastrointestinal and hematological toxicities (Kirwan *et al.*, 2003). Natural products offer an excellent alternative for therapeutic use as opposed to synthetic compounds because of their relatively well established safety profile. Several natural products are being tested as potential radio-sensitizers.

Berberine is a natural compound that allows prevention, suppression and retardation of carcinogenesis. Berberine [1,7-bis-(4–Hydroxy-3-methoxyphenyl) -1,6 heptadiene-3,5-dione] is a major constituent of product extracted from the rhizome of the plant *Berberisaristata* found in south and south-east tropical Asia. Berberine has been shown to be a potent chemopreventive agent inhibiting tumor progression against skin, oral, intestinal, breast, colon and prostate cancer (Anand *et al.*, 2008). Berberine has been shown to confer radiosensitizing effect in prostate cancer cells, squamous cell carcinomas (Chendil *et al*., 2004; Khafif *et al*., 2005) and HeLahuman cervical cancer cells (Javvadi *et al.*, 2008).

The major problem with cervical cancer is that the cancer cells become increasingly radio-resistant due to activation of various antiapoptotic genes/cascades resulting in therapy failure, and the standard chemoradiotherapy regime is unable to address this problem (Aggarwal *et al*., 2006). The goal of this study was to compare the standard chemoradiotherapy regime comprising of cisplatin/IR with the cisplatin/Berberine/IR combination therapy and assess its effect on protein which confers radio-resistance to cervical cancer cells *in vitro*.

Materials and methods

Cell culture and chemicals: Human cervical cancer HeLa, cells were obtained from National Centre for Cell Sciences, Pune, India and were maintained in either Dulbecco's modified Eagle's medium or RPMI1640 (Sigma, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Hyclone), antibiotics, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were exposed to varying IR. Antibodies against p73, Bclxl, Bax, Cyclin D1, AIF, Cox 2 as well as secondary AP conjugated antibodies were obtained from Santa Cruz, USA. Berberine was obtained from Sigma, USA.

Flow cytometery: Cells (1 x 10^4 cells) were treated with 50 µM and 75 µM Berberine for 24 h and then harvested. Cells were fixed in 70% ethanol and left overnight at -20°C. Cells were then washed with PBS and incubated in staining solution (20 µg/mLpropidium iodide, 50 µg/mL RNase, 0.1% Triton X-100 and 0.1 mM EDTA) for 2 h at 4°C in dark. The DNA content of the cells was measured by flow cytometer (Becton Dickenson, USA) using Diva software.

Assay of telomerase activity: Telomerase activity was measured using the PCR-ELISA kit based on TRAP assay (Roche Molecular Biochemicals, Germany). The samples were lysed and an aliquot containing 2 µg protein was used for the assay. Telomerase positive embryonic kidney cell line (HEK-293) was used as positive control while heat inactivated HeLa extract was used as negative control. The telomerase activity was detected and expressed as relative units (RU) (Khanna *et al*., 2003).

Western blot analysis: The level of expression of various proteins was determined in control and treated cells by Western blotting as described previously (Singh *et al*., 2007). Briefly, cells were washed twice in PBS and lysed in RIPA buffer. Total protein was determined by the Bradford assay (1976). Equal amount of protein was loaded and run on 10-15% SDS-Polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA, followed by hybridization with respective primary and secondary antibody. Final detection was performed with BCIP/NBT substrate (Promega, USA). The bands were analyzed and quantified using ά image scanner densitometer and normalized with β-actin control. The density of control was taken as 1 and results of treatment were expressed in relation to the control as relative unit (RU).

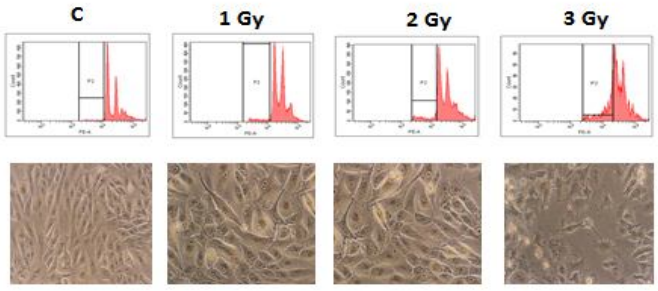
Assay of caspase-3, -9 activity: Caspases-3 and -9 activity were measured by the direct assay for caspase enzyme activity in the cell lysate using synthetic fluorogenic substrate (Ac-DEVD-AFC; substrate for caspase 3; MBL Bioscience, USA; Ac-LEHD-AFC, substrate for caspase 9; Genotech, USA) as described by the manufacturer. Amount of fluorogenic AMC/AFC moiety released was measured using a spectrofluorimeter (ex. 380 nm, em. 420-460 nm for Caspase-3; ex. 400 nm, em. 490-520 nm for caspase-9). The results are expressed in arbitrary fluorescence units/mg protein (Singh and Singh, 2008).

Statistical analysis: Results were expressed as mean of three individual experiments. Standard deviation was calculated using Microsoft excel.

Results

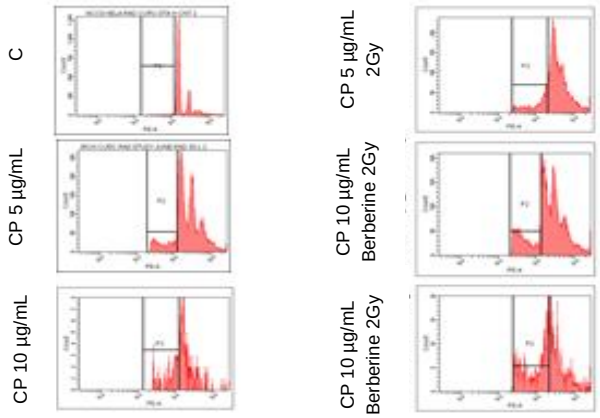
Ionizing radiation resulted in increase in apoptosis in cervical cancer cells with increasing radiation dose: For treatment with ionizing radiation, HPV 18 positive HeLa positive cell line is chosen. Cells were treated with ionizing radiation doses ranging from 1Gy to 3Gy, to assess their toxicity of this dose on the cell line. Gradual increase in apoptosis was found on increasing radiation dose from 1Gy to 3Gy (Fig. 1). Our results indicates that cervical cancer cell line HeLa is resistant to IR dose of 1Gy resulting in 3.8% apoptosis, which is similar to the controls (3.6%) demonstrating its radio-resistance at this dose.

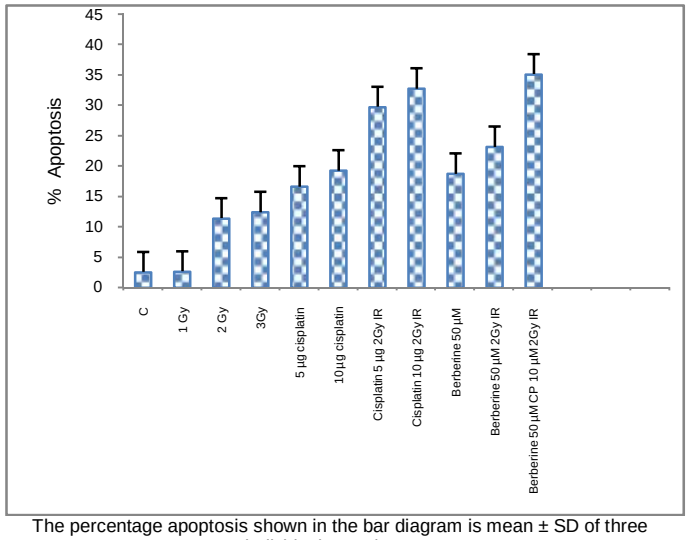
Fig. 1. Microscopic and flow cytometric analysis of apoptosis in HeLa cells on treatment with IR (1-3Gy).



Treatment with 2Gy radiation dose resulted in increase in apoptosis to 11.45% apoptosis. Escalation of radiation dose to 3Gy resulted in marginal increase in apoptosis to 12.45% in HeLa cells. Since, there was only a marginal increase in apoptosis on increasing radiation dose from 2Gy to the dose of 3Gy, hence we settled for the radiation dose of 2Gy for all the experiments. This dose was used in combination treatments with cisplatin and berberine also.

Fig. 2. Microscopic and flow cytometric analysis of apoptosis in HeLa cells on treatment with cisplatin/IR and 50 µM Berberine/10 µgM cisplatin/IR for 24 h.





The percentage apoptosis shown in the bar diagram is mean ± SD of three individual experiments.

Cisplatin resulted in enhanced apoptosis in HeLa cell lines by itself and in combination with IR: Platinum compounds like cisplatin in combination with radiotherapy are used extensively for treatment of cervical cancer, so we tried to mimic this situation *in vitro* on cervical cancer cell line HeLa. First we assessed the effect of cisplatin alone on this cell line and in combination with IR (2Gy). Treatment of HeLa with 5 µg/mL cisplatin for 24 h resulted in 16.75% apoptosis respectively (Fig. 2). Coupling this dose of cisplatin with 2Gy IR resulted in increase in apoptosis to 19.3% in HeLa cells respectively. Escalation of cisplatin dose to 10 µg/mL for 24 h resulted in increase in apoptosis in HeLa to 29.8% and in combination with 2Gy IR to 32.8%. Thus, a chemoradiation dose of 10 µg/mL CP/2Gy IR was selected for further experiments (Fig. 2).

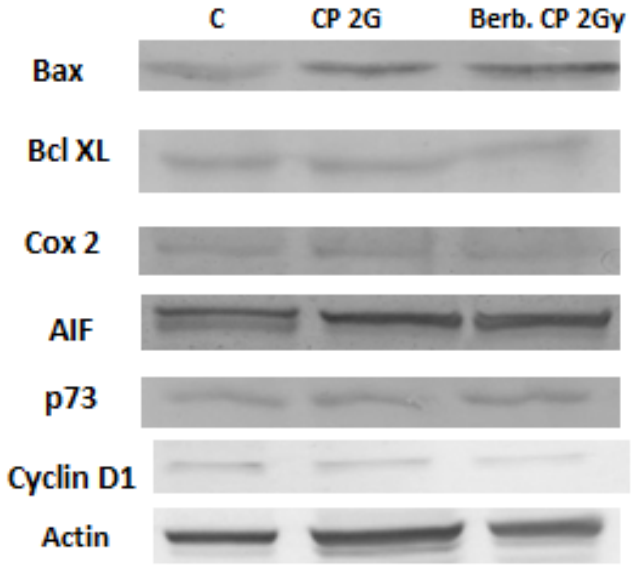
Coupling of berberine to 10 µg/mL CP/2Gy chemoradiation dose resulted in marked increase in apoptosis in HeLa: Berberine has been established as a potential chemotherapeutic agent in various clinical trials and has been found to be well tolerated at higher doses but its bioavailabilty remains a major problem. Howell *et al*. (2007) have summarized based on *in vitro* and *in vivo* studies and clinical trials on natural products like curcumin and berberine that the concentrations of natural products that were achievable in the plasma of patients were only at a lower micromolar range; hence, they have suggested that for *in vitro* studies, concentration of berberine in less than 50 µM range do not have any physiological relevance.

The significant radiosensitization achieved by moderate dose of Berberine at relevant doses *in vitro* (3-6Gy) has promising implications for improving radiation therapy especially in radio-resistant tumors such as the tumors of the uterine cervix. Hence, we coupled the above dose of 10 µg/mL CP with 50 µM berberine. The cells were treated to this combination dose for 24 h followed by 2Gy IR. An abrupt increase in apoptosis was seen in the HeLa cell line showing 46.35% apoptosis which was higher than 32.8% apoptosis observed with 10 µg/mL CP/2Gy chemo-radiation dose, used as standard therapy (Fig. 3).

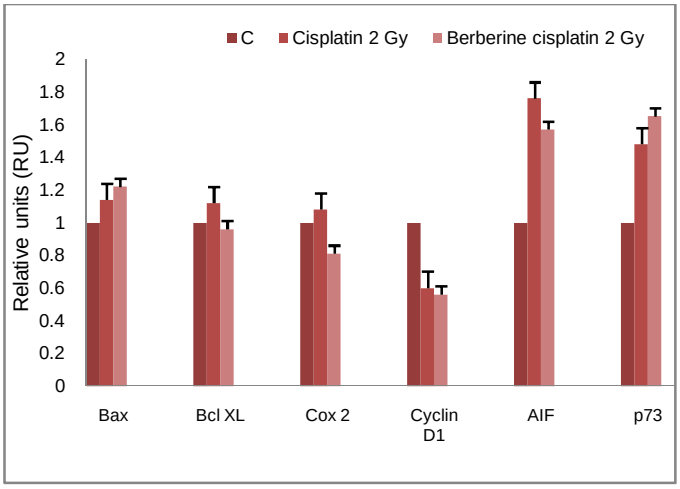
Clearly our results show that berberine is selectively increasing apoptosis in the cell line. We then compared the effect of 50 µM Berberine/10 µg/mL CP/2Gy combination with 10 µg/mL CP/2Gy on various proteins involved in apoptosis both pro-apoptotic and anti-apoptotic, radio-resistance and inflammatory response (Fig. 2).

Combination of berberine and cisplatin results in increased expression of p73 as compared to cisplatin based radiotherapy: p53 protein is a well-known tumor suppressor protein that functions primarily as a transcription factor, initiates cell cycle arrest and apoptosis after genotoxic stress.

Fig. 3. Effect on the level of Proapoptotic Bax, inflammatory COX-2, antiapoptotic Bcl-XL, apoptotic AIF, cyclin D1 and p73 protein in HeLa cells by western blotting.



Lane 1: Control, Lane 2: Cisplatin 10 µg for 24 h followed by 2Gy IR, Lane 3: Cisplatin 10 µg/50 µM Berberine for 24 h followed by 2Gy IR (RU).



The cell line used in the study i.e. HeLa is HPV (Human Papilloma Virus) positive cell line which encodes for viral protein E6 which causes ubiquitin mediated degradation of p53 so another member of p53 family p73 becomes important in these cells. It has been shown previously that p73 plays an important role in hydrogen peroxide induced apoptosis (Singh *et al*., 2007; Singh and Singh, 2008). Since, radiation induced apoptosis involves ROS, so we determined the effect of cisplatin and cisplatin/berberine/IR combination on p73 in the cell line. In HeLa cells, there was a 34% increase in p73 expression on treatment with cisplatin/IR but on treatment with berberine/cisplatin/IR the expression of p73 increased to 44% demonstrating that p73 responds to berberine (Fig. 3).

Javvadi *et al*. (2008) have shown that ROS plays an important role in curcumin/IR mediated apoptosis in Hela cells. Our results on other natural product berberine are in agreement with their findings and suggest that cisplatin/berberine/IR induced activation of p73 which may involve reactive oxygen species (ROS).

Berberine/cisplatin/IR combination treatment resulted in activation of proapoptotic Bax and reduction in level of Bcl XL: Cells on exposure to IR along with chemotherapeutic agent results in DNA damage and if this is severe, p53 may trigger programmed cell death by means of proapoptotic genes such as Bax and inhibition of antiapoptotic Bcl XL. It has been demonstrated that radio-resistant laryngeal cancer was associated with increased Bcl-2 and Bcl-XL expression and loss of Bax expression. Bcl-2 family has been proposed to predict radiotherapy outcome (Nix *et al*., 2005). The association between expression of Bcl-2, Bcl-XL and Bax with radio-resistant cancer suggests a potential mechanism by which cancer cells avoid the destructive effects of radiotherapy. We probed the effect of berberine/cisplatin/IR combination on level of Bax in HeLa cells. The Bax expression was 14% on treatment with cisplatin/IR, but this increased to 22% with respect to control on treatment with berberine/cisplatin/IR combination, However, in case of antiapoptotic Bcl-XL we obtained a 12% increase in expression in HeLa cells on treatment with cisplatin/IR (Fig. 3). In contrast, on treatment with berberine/cisplatin/IR we obtained no notable change in level of BclXL.

Berberine/cisplatin/IR combination resulted in decrease in expression of cyclin D1 in HeLa cells: Cyclin D1 is involved in cell-cycle arrest in DNA-damage response. Cyclin D1 has been shown to be induced by low-dose ionizing radiation in human keratinocytes with an adaptive radio-resistance (Ahmed *et al*., 2008). On exposure of HeLa to cisplatin/IR there was a 40% decrease in expression of cyclin D1 which was reduced to 44% on treatment by berberine/cisplatin/IR (Fig. 3).

Berberine/cisplatin/IR combination treatment did not affect the expression of COX-2 and AIF in comparison to cisplatin/IR: COX-2 has been implicated in carcinogenesis of systemic cancers. COX-2 inhibition has been shown to increase the radio-sensitivity of various tumors. Results from this study demonstrate that on treatment of HeLa cells with cisplatin/IR there was an 8% increase in expression of COX-2 but treatment of these cells with berberine/cisplatin/IR resulted in 19% decrease in expression of COX-2. These results indicated that though berberine/cisplatin/IR based therapy is repressing COX-2, the effect is only marginal. AIF expression was unaltered remaining nearly the same on both the treatments in HeLa cell line (Fig. 3), suggesting its non-involvement.

Berberine/cisplatin/IR treatment enhances activation of both Caspase-3 and -9: Caspase-3 and -9 have been implicated to play an important role in mitochondrial mediated apoptosis by causing activation of caspase activated DNAse, finally causing degradation of DNA. Hence, we compared the effect of berberine/cisplatin/IR and cisplatin/IR on caspase-3 and -9 activities. The results show that in HeLa cells there was 1.07 fold increases in the activity of caspase-3 on treatment with cisplatin/IR, while there was a 1.83 fold increase on treatment with berberine/cisplatin/IR (Fig. 4a). Clearly berberine is causing activation of caspase-3. To assess whether enhanced apoptosis was being mediated through mitochondrial pathway, we also studied the effect on caspase-9 activity. HeLa cells showed 0.59 fold increase in activity of caspase-9 on treatment with cisplatin/IR while there was a 0.81 fold increase on treatment with berberine/cisplatin/IR. Thus, berberine appears to be mediating apoptosis through mitochondrial pathway (Fig. 4b).

Fig. 4a. Caspase-3 activity after treatment with CP 10 µg for 24 h followed by 2Gy IR and 10 µg CP/50 µM berberine for 24 h followed by 2Gy IR in HeLa cells.

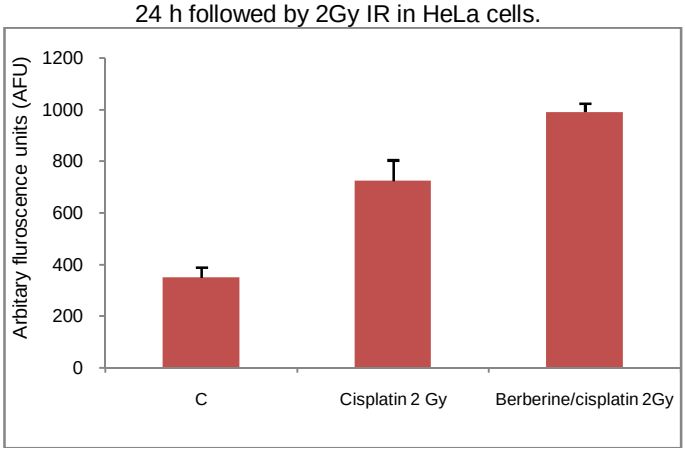


Fig. 4b. Caspase-9 activity after treatment with CP 10 µg for 24 h followed by 2Gy IR and 10 µg CP/50 µM berberine for 24 h followed by 2Gy IR in HeLa cells.

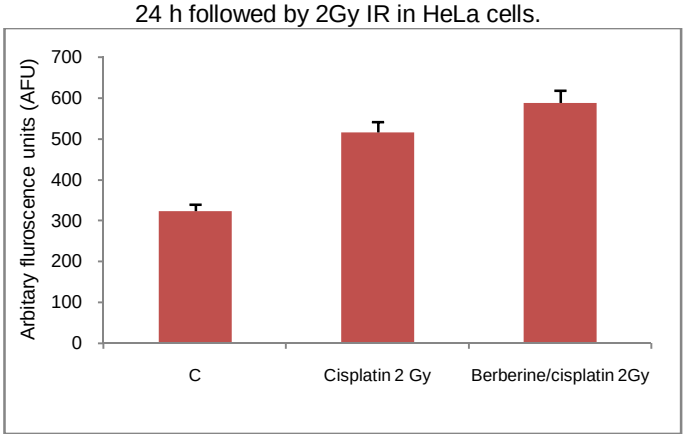
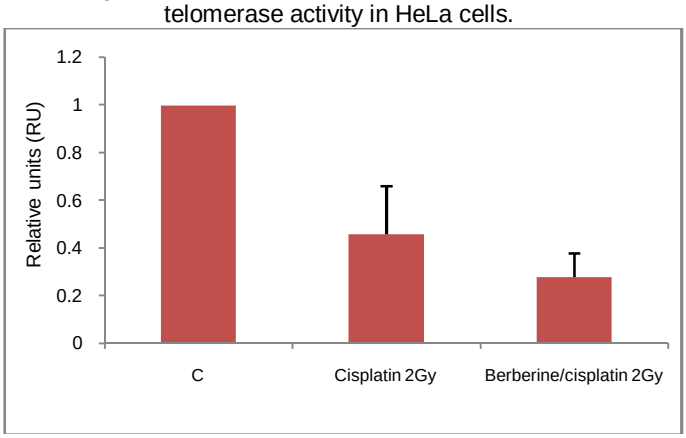


Fig. 5. Effect of 10 µg CP for 24 h followed by 2 Gy IR and CP 10 µg/50 µM berberine for 24 h followed by 2Gy IR on telomerase activity in HeLa cells.



Berberine/cisplatin/IR combination causes reduction in activity of telomerase with respect to cisplatin/IR: Telomerase activation plays a critical role in tumor growth and progression, in part by maintenance of telomere structure. Indeed, the ubiquitous expression of telomerase in human cancers makes telomerase a promising target for cancer therapy. We assessed the effect of berberine/cisplatin/IR combination dose on telomerase with respect to cisplatin/IR combination dose by using PCR/ELISA methodology which assesses activity of hTERT. We observed that on treatment of HeLa there was 45% decrease in telomerase activity with cisplatin/IR while there was a 66% decrease in telomerase activity on treatment with berberine/cisplatin/IR (Fig. 5). The findings suggests that berberine based therapy causes substantial decrease in telomerase activity and this therapy can be a potent telomerase targeted approach for treatment of cervical cancer.

Discussion

Chemo/radiotherapy regime which is cisplatin based radiotherapy is used for the treatment of advanced cervical cancer in India. Evidence shows that most of the chemotherapeutic drugs used in current clinical practice are radio-sensitizers. Several newer cytotoxic agents with radiosensitizing properties are being tried in combination with cisplatin/IR but their use is generally limited by dose related toxicities (Candelaria *et al.*, 2006). Natural products open up all new avenues for treatment of cancer as they are generally tolerated at high doses. Animal studies have confirmed the antitumorigenic activity of natural products like berberine, curcumin, withaferin (Howells *et al*., 2007).

Phase I clinical trials on curcumin showed that it is safe to humans up to 12,000 mg/d when taken orally (Cheng *et al*., 2001; Sharma *et al.*, 2001; Lao *et al.*, 2006) and caused histological improvement of precancerous lesions in patients, suggesting that it is biologically active at these doses (Cheng *et al*., 2001).

Previous reports have indicated that berberine confers radio-sensitizing effects in prostate and squamous cell carcinoma cell lines and recently in cervical cancer cell lines HeLa and SiHa. Thus, we evaluated the effect of berberine in combination with cisplatin/IR treatment. Our results suggest that berberine/cisplatin/IR based therapy is likely to be more effective and safer to treat cervical cancer, as berberine is well tolerated in humans, even at high doses. We present *in vitro* evidence that this approach targets radio-resistance and antiapoptotic proteins in a much more specific way compared to the standard cisplatin/IR based therapy. When cells are exposed to clinically relevant doses of ionizing radiation it causes DNA damage by generation of reactive oxygen species (ROS). This DNA damage causes a rapid ROS dependent activation of proapoptotic and antiapoptotic cascade which involves activation of p53 family of genes acting downstream to trigger apoptosis if the damage is not repairable.

The tumor cells are dynamic with respect to their reliance on specific cell signaling pathway to exist and rapidly adapt to repeated toxic challenges in an attempt to maintain tumor survival. Prolonged inhibition of any one of these pathways however, gives rise to lineage of cells which become resistant to inhibitor drug, with point mutations in the specific targeted proteins, or by reprogramming of multiple signaling processes within the cell (Valerie *et al*., 2007). So the need for today is the therapies which target these multiple pathways and result in selective apoptosis of cancer cells and thus, deal with the problem of radio-resistance. The cell line used in this study is HPV positive, in which wild type p53 is targeted for ubiquitin mediated degradation so other members of p53 family like p73 become particularly important in the cell line (Singh *et al*., 2007; Singh and Singh, 2008). We observed that the activation of p73 in cisplatin/IR treated sample but this activation was much more in berberine/cisplatin/IR treated samples which indicates that combination of berberine is causing much more activation of p73 probably through mediation of ROS. This has been demonstrated recently in some studies in which natural products like berberine were shown to be a pro-oxidant in combination with IR and its radio-sensitizing properties were attributed to ROS mediated signaling (Javvadi *et al*., 2008). Bax and Bcl-XL are members of Bcl-2 family of proteins which control apoptosis and are associated with regulating the mitochondrial membrane permeability. Proapoptotic proteins like Bax by translocation from the cytosol to the mitochondria induce cytochrome c release, whereas, Bcl-XL exerts its anti-apoptotic activity, at least in part by inhibiting the translocation of Bax to the mitochondria (Mohammad *et al*., 2008; Ow *et al*., 2008). Our results show an increase in Bax and a decrease in Bcl-XL on berberine treatment in HeLa cell line. AIF (Apoptosis inducing factor) remained unchanged thereby suggesting its non-involvement.

Caspases, a family of aspartic acid-specific proteases, are the major effectors of apoptosis. Once activated, caspases preside over the ordered dismantling of the cell through restricted proteolysis of hundreds of substrate proteins (Ow *et al.*, 2008). Caspase-3 has been implicated in both the extrinsic and intrinsic pathway of apoptosis. Our findings show activation of caspase-3 on both the treatments but this activation was more marked on berberine/cisplatin/IR treatment implicating that cells are undergoing apoptosis through caspase-3 mediated pathway. Similarly, caspase-9 was activated on both the treatments in HeLa cells, but was more marked on berberine/cisplatin/IR treatment, implicating involvement of mitochondrial mediated apoptosis pathway.

Next, we assayed the effect of berberine/cisplatin/IR on proteins involved in radio-resistance like telomerase, cyclin D1 and Cox-2. Cyclin D1 is involved in cell-cycle arrest in DNA-damage response. Cyclin D1 contributes to regulate G1 progression by forming a complex with different cyclin-dependent kinases. It has oncogenic properties and is frequently over expressed in several human tumor types. Cyclin D1 has been shown to be induced by low-dose ionizing radiation in human keratinocytes with an adaptive radio-resistance (Ahmed *et al*., 2008). Our results show a similar reduction in level of cyclin D1 on berberine treatment in the HeLa cell line.

Cyclooxygenase-2 (COX-2), an enzyme induced by proinflammatory cytokines, mitogenic substances, oncogenes, growth factors, and hypoxia, among others, is involved in the metabolic conversion of arachidonic acid to prostaglandins in inflamed tissues and neoplasia. COX-2 is often over expressed in malignant tumors and premalignant lesions. Because COX-2 may also be a determinant of tumor radio-resistance, its inhibition or inhibition of its products (prostaglandins) may improve tumor response to radiotherapy. Analysis of the effect of cisplatin/IR show that COX-2 was elevated in HeLa cells on treatment with cisplatin/IR indicating that this radio-resistant marker is elevated in the cell line however on treatment with berberine/cisplatin/IR COX-2 was down regulated in the HeLa cell line showing maximum inhibition.

Telomeres and telomerase play a role in the regulation of the life span of the cell. Human cells express low levels of telomerase; however when telomere length reaches a critical level, abnormal activation of telomerase can lead to immortalization and uncontrolled proliferation (Gandellini *et al.*, 2007; Agarwal *et al.*, 2008). Our result shows that berberine treatment caused a substantial reduction in telomerase activity. Thus, berberine is conferring a selective advantage over cisplatin based radiotherapy by causing marked decrease in telomerase activity.

Conclusion

Our findings provides *in vitro* evidence that supports the clinical importance of coupling berberine with cisplatin as an efficient radio-sensitizer for treatment of cervical cancer as it causes enhanced activation of p73 causing further down regulation of antiapoptotic Bcl-XL and activation of caspase-3 and -9, resulting in enhanced apoptosis. We also provide evidence that berberine/cisplatin based radiotherapy causes substantial deregulation of telomerase activity, cyclin D1 and COX-2, thus overcoming partially the effect of radio-resistance by acting as a potent radio-sensitizer. This data has clinical relevance as it highlights the importance of overcoming the problem of radio-resistance by specifically targeting radio-resistant proteins using Berberine which is well tolerated in human beings even at high doses, and thus, promises to be an effective drug in future clinical trial along with cisplatin/IR.

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