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Suppression of Telomerase Activity by Pyrimethamine: Implication to Cancer

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ABSTRACT

Background: Although pyrimethamine (Tindurin™) appears to be effective in the prevention and treatment of some infectious diseases, very little information exists on its unpredictable properties. We design this study to evaluate its anti-tumoral effect on a model of cell line. **Methods:** The cytotoxic influence of Pyrimethamine on prostate cell line was investigated using an *in vitro* colometric assay. The potential modulatory effects on metastasis, apoptosis, and immortality characteristics of cells were assessed with gelatin zymography, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and telomeric repeat amplification protocol, respectively. **Results:** Cytotoxicity analysis of pyrimethamine revealed a dose-dependent fashion. An apoptotic influence of pyrimethamine was also confirmed by data obtained from TUNEL assay. Dose-dependent inhibitory effect on matrix metalloproteinases (MMP) was seen in pyrimethamine. A potent inhibitory effect of pyrimethamine was also established by data achieved from TRAPeze telomerase detection kit. **Conclusions:** Collectively, as induction of apoptosis together with MMP and telomerase inhibition could be indicative of cancer treatment, pyrimethamine might be considered as a chemopreventative agent in cancer. *Iran. Biomed. J. 11 (4): 223-228, 2007*

Keywords: Telomerase, Cancer, Matrix metalloproteinases (MMP), Pyrimethamine

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INTRODUCTION

Despite several studies on molecular researches on the basis of neoplastically transformed cell, cancer remains a major cause of morbidity and mortality in human. The finding of new components is imperative, and telomerase inhibitors have the potential to provide an additional option for chemotherapy. Telomerase is a ribonucleoprotein that synthesizes and directs the telomeric repeats onto the 3’ end of existing telomeres using its RNA component as a template [1, 2]. Recent studies have shown that many proliferating tumor cells retain a certain level of telomerase activity [3-5].

Another critical event that takes place during tumor cell invasion and metastasis is basement membrane and extracellular matrix degradation by proteolytic enzymes. Several proteases are secreted by invading neoplastically transformed cells, such as serine proteases, plasminogen activators and matrix metalloproteinases (MMP) [6, 7]. The elevated levels of MMP have been shown in many tumors having strong association with the invasive phenotype [8-11]. Thus, each component with potential inhibitory influence on MMP expression and telomerase activity is able to reduce the risk of cancer.

Pyrimethamine (2, 4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine), a folic acid antagonist, is extensively used in the treatment and prophylaxis of opportunistic infections such as malaria and toxoplasmosis. It exerts its activity by inhibiting plasmodial dihydrofolate reductase (DHFR), thus indirectly blocking the synthesis of nucleic acid [12, 13]. In our Previous studies to investigate the therapeutic effect of pyrimethamine, suppression of MMP type 2 (MMP-2) activity and induction apoptosis were observed *in vitro* fibrosarcoma and *in vivo* collagen-induced arthritis models [14-16].

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In this study, we assess the modulatory effects of pyrimethamine on telomerase and MMP activity in cancer cells. We observed that pyrimethamine inhibits telomerase and metalloproteinases as well as promotes cell death through apoptosis. Inhibition of cell proliferation suggests that pyrimethamine is a viable approach to antitelomerase therapy, and our results should encourage and guide further testing of other small molecules to modulate telomerase activity.

MATHERIALS AND METHODS

Cell culture. The prostate cancer cell line (PC-3) was seeded at initial density of 2×10^4 cells/well in 96-well tissue culture plates. Cells were maintained in DMEM medium supplemented with 5% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 µg/ml, under 5% CO_2, and saturated humidity at 37°C.

Dose-response analysis. Pyrimethamine (Tindurin) (Sigma-Aldrich, Taufkirchen, Germany) was freshly prepared in DMSO (Sigma-Aldrich, Taufkirchen, Germany) before being transferred into overnight cultured cells. The final concentration of DMSO in the medium was 1/1000 (v/v). Pentaplicate confluent cells were treated with 0.1, 1, and 10 µg/ml concentrations of Pyrimethamine solution. Non-treated cells were used as control. Cells were cultured overnight and were then subjected to colorimetric assay. A sample of the media was used for zymoanalysis. Paralleled cultures were also used for apoptosis and telomerase activity assays.

Colorimetric assay. This technique was used according to the method published by Saadat *et al.* [17]. Briefly, after each experiment, the cells were washed three times with ice-cold PBS, followed by fixation in a 5% formaldehyde solution. The fixed cells were washed three times and stained with 1% crystal violet. The stained cells were washed, lysed and solubilized with 33.3% acetic acid solution. The density of developed purple color was read at 580 nm.

Zymoanalysis. This assay was done according to Khorramizadeh *et al.* [18]. The technique was used for the detection of gelatinase (collagenase type IV or MMP-2) and MMP-9, in conditioned-media. Briefly, aliquots of conditioned media were subjected to electrophoresis in (2 mg/ml) gelatin containing polyacrylamide gels, in the presence of SDS-PAGE under non-reducing conditions. The gels underwent electrophoresis for 3 hours at a constant voltage of 80 volts. After electrophoresis, the gels were washed and gently shaken in three consecutive washings in 2.5% Triton X-100 solution to remove SDS. The gel slabs were then incubated at 37°C overnight in 0.1 M Tris HCl gelatinase activation buffer (pH 7.4) containing 10 mM $CaCl_2$ and subsequently stained with 0.5% Coomassie Blue. After intensive destaining, proteolysis areas appeared as clear bands against a blue background. Using a UVI Pro gel documentation system (GDS_8000 System), quantitative evaluation of both surface and intensity of lysis bands, on the basis of grey levels, was compared relatively to non-treated control wells and expressed as “Relative Expression” of gelatinolytic activity.

Cell apoptosis assay. This assay was carried out based on our previous publication with slight modifications [19]. Briefly, cells were treated with different agents for 24 h and then fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for detecting DNA fragmentation was performed by flowcytometric analyze as indicated by kit instructions. (APO-BRDU, Roche, CA, USA). The cell nuclei were stained with fluorescein and propidium iodide and apoptotic and total cells were counted by flowcytometry instrument (FACSCalibar Becton Dickinson, USA). The results were expressed as percentage of apoptotic cells.

Detection and measurement of telomerase activity. Telomerase activity was measured by telomere repeat amplification protocol using the TRAPeze telomerase detection kit (Intergen, Inc., USA) [20]. Briefly, equal number of cells was lysed using CHAPS buffer provided by the kit supplier. The telomerase activity in the cell lysate was then prompted by incubating with a substrate oligonucleotide (TS primers) at 30°C for 30 min. Telomerase adds a number of telomeric repeats (GGTTAG) to the 3’ end of TS primer. The extended oligonucleotides were the amplified with a two-step 30 cycles PCR (94°C/30 seconds, 59°C/30 seconds). The signature telomerase “DNA laddering” with six base increments starting at 50 nucleotides was visualized on a native 12%

polyacrylamide gel, followed by silver nitrate staining of the gel. Telomerase activity was calculated by the ratio of the intensity of telomerase ladders to the intensity of 36-bp internal standard (SC band), as assessed by arbitrary units. Percentage of inhibition was calculated by comparing telomerase activity of pyrimethamine-treated cells with that of non-treated control cells. The levels of telomerase activity were within the linear range of the TRAP assay.

Statistical analyses. The differences in cell proliferation, gelatinase activity, nitric oxide level, telomerase activity and programmed cell death were compared using the Student's *t* test. *P* values <0.05 were considered significant.

RESULTS

Effect of pyrimethamine on cell biocompatibility. Cell cytotoxicity of pyrimethamine is shown in Figure 1. Treatment of cells with pyrimethamine (0.1, 1, 10 µg/mL) induced morphological changes and inhibited the growth of PC-3 cell lines. The cytotoxic effects of pyrimethamine caused 3.72%, 19.01% and 24.421% cell death at 0.1, 1 and 10 µg/mL, respectively, as compared to that of non-treated cells as control.

Effect of pyrimethamine on the modulation of MMP-2 production. We studied the different pyrimethamine concentrations in modulating MMP-2 gelatinolytic activity in PC-3 cells. Pyrimethamine decreased MMP-2 gelatinolytic activity in a dose-dependent manner after a 24-h incubation in serum free media. This effect was significant in doses equal or greater than 1 µg/ml ($P<0.05$ vs control) (Fig. 2).

Effect of pyrimethamine in apoptosis. Using the TUNEL assay method, we determined the rate of apoptosis of pyrimethamine-treated cells in comparison with positive, negative and non-treated cells. As depicted in Figure 3, pyrimethamine with concentrations 0.1 µg/mL, 1 µg/mL, and 10 µg/mL caused 0.69%, 7.22% and 9.24% programmed cell death as normalized against un-treated control cells, respectively.

Inhibition of telomerase activity by pyrimethamine. Various concentrations (0.1, 1, and 10 µg/ml) of pyrimethamine were used to investigate the correlation between pyrimethamine treatment and telomerase activity. Pyrimethamine decreased telomerase activity in a dose-dependent manner as depicted in Figure 4. The results were significant at all concentrations versus non-treated control cells. ($P<0.05$).

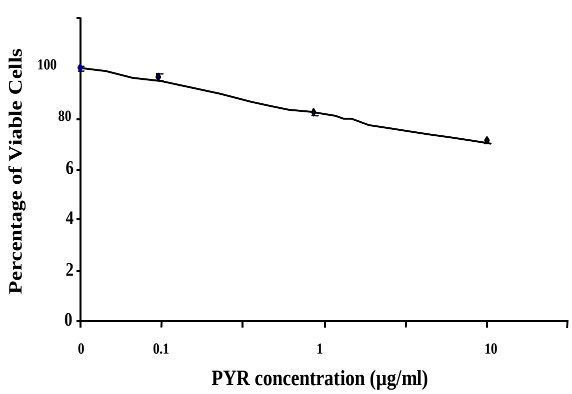


Fig. 1. Cytotoxic analysis of pyrimethamine (PYR). Cell survival of prostate cancer (PC)-3 cell line was tested against PYR in pentaplicate manner. PC-3 as a sensitive cell line, showed more tolerability against increasing amounts of PYR.

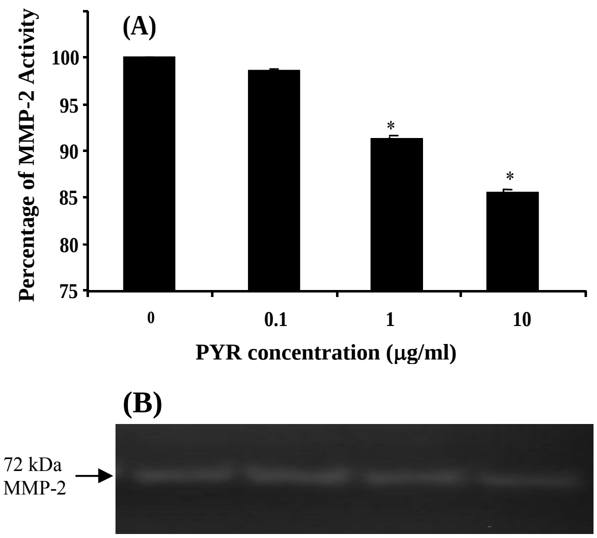
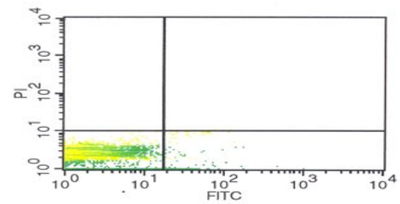
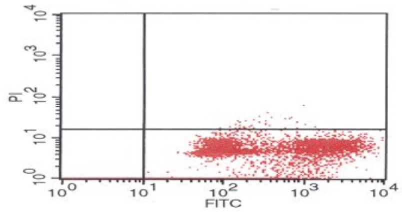


Fig. 2. The inhibitory effect of pyrimethamine (PYR) on matrix metalloproteinases (MMP)-2 activity. Prostate cancer cells (PC-3) (2×10^4 cell/well) were incubated for overnight with increasing dose of PYR as described in Materials and Methods. Non-treated cells were used as controls. Analyzes were performed using UVI Pro Gel Documentation system (Cambridge, UK). Surface and intensity of lyses bands on the basis of grey level were analyzed. PYR-treated cells were investigated in triplicate. **(A)**, Analysis of densitometric data as depicted in percentage of MMP-2 activity in treated cells vs. untreated cells; **(B)**, Demonstration of a representative zymogram gel. From left to right: Untreated control cells; cells treated with 0.1, 1, and 10 µg/ml pyrimethamine, respectively.

(A)



Quad	Events	%Gated
UL	0	0.00
UR	7	0.11
LL	5940	97.23
LR	162	2.65



Quad	Events	%Gated
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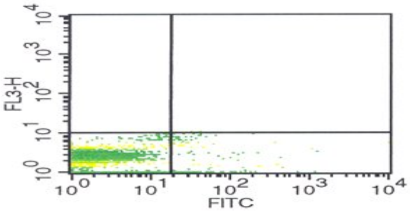
UR	48	0.55
LL	264	3.04
LR	8377	96.41



Quad	Events	%Gated
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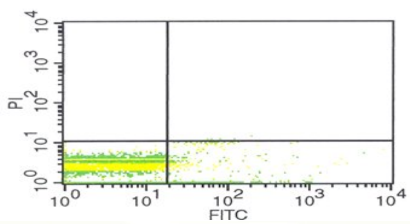
UR	2	0.04
LL	5274	96.66
LR	180	3.30



Quad	Events	%Gated
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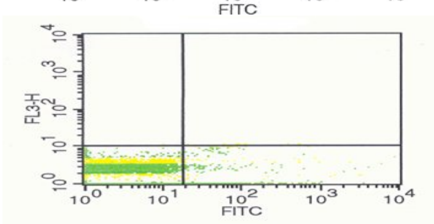
UR	12	0.19
LL	5716	90.36
LR	596	9.42



Quad	Events	%Gated
UL	0	0.00
UR	15	0.23
LL	5650	88.45
LR	723	11.32

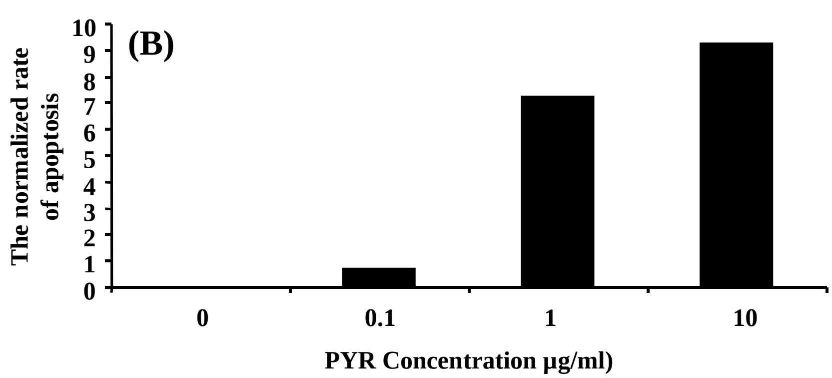


Fig. 3. Assessment of apoptosis by flowcytometry at various concentration of pyrimethamine (PYR). Figures were normalized against non-treated cells. **(A)**, From up to down: non-treated cells as control, apoptosis positive control (96.41% apoptotic), cells treated with PYR at 0.1 µg/ml, 1µg/ml, and 10 µg/ml concentrations Apoptosis Flowcytometry Charts: "X" axes, FITC, "Y" axes, Propidium Iodide(PI), fluorescence reported in arbitrary units.; **(B)**, Depiction of the normalized rate of apoptosis in pyrimethamine-treated cells. From left to right: non-treated prostate cancer (PC)-3 cells (97.23% viable, 2.65% apoptotic), 0.1 µg/ml (3.30% raw data, 0.69% normalized apoptosis rate), 1 µg/ml (9.42% raw data, 7.22% normalized apoptosis rate), and 10 µg/ml (11.32% raw data, 9.24% normalized apoptosis rate) PYR-treated PC-3 cells.

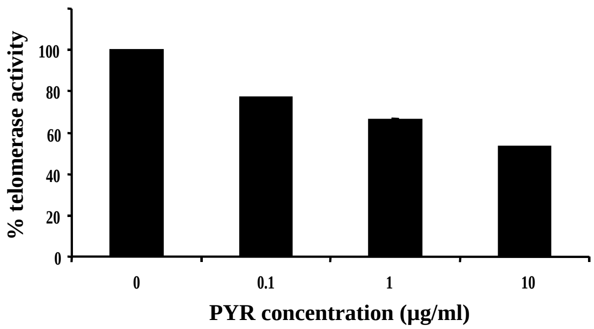


Fig. 4. Inhibition of telomerase activity in prostate cancer-3 cells by pyrimethamine (PYR) as detected by the TRAP assay. Telomerase activity was quantitated as described in Material and Methods. PYR decreased telomerase activity in a dose-dependent manner. The results were significant at all concentrations versus non-treated control cells ($P<0.05$).

DISCUSSION

The mechanisms by which pyrimethamine exert its anti-tumor effects are postulated to involve cell cycle arrest, anti-angiogenic effects, induction of apoptosis and inhibition of MMP [21]. A crucial feature of malignant cancer cell is immortality and disruption of extracellular matrix. Modulation of the molecules involves in these processes is a main target of cancer research. In the present study, we attempted to cast a light on the unexpected potential therapeutic effects of pyrimethamine on PC-3.

To determine whether inhibition of telomerase would ultimately limit proliferation, we treated PC-3 cells with pyrimethamine. According to our findings, pyrimethamine decreased telomerase activity in a dose-dependent manner. Typically, when a supposed anti-proliferative agent is applied to cells, an effect is observed within hours or days. Telomerase is an unusually difficult target for drug discovery, because a cellular response that depends on telomere shortening will require weeks to become apparent. However, DNA fragmentation, a typical feature observable in cells undergoing apoptotic cell death, was induced.

The Data obtained from TUNEL assay showed that induction of cell apoptosis by pyrimethamine is a dose-dependent fashion. Recent work has shown induction of peripheral blood lymphocyte apoptosis, supporting our records [22]. Moreover, our data were in agreement with our cytotoxicity experiments which demonstrated that pyrimethamine is able to decrease in proliferation of the PC-3 cell line. However, molecular mechanisms by which pyrimethamine induces apoptosis in tumor cells is still unknown. Besides, as one of the critical steps for tumor invasion and metastasis is the destruction of extracellular matrix which is catalyzed mainly by the MMP [23-25], the inhibition of MMP could be beneficial in preventing tumor metastasis. Only limited information is available on the effects of DHFR inhibitors in regulating MMP activity and/or production. Our zymography analysis test of pyrimethamine showed that modulation of MMP activity is associated with increasing concentration of this drug (Fig. 2).

With this regard, potential ability of this agent to inhibit MMP could be an extra characteristic to prevent tumor invasion and metastasis. Collectively, pyrimethamine might be assumed as a component, which has the potential to be applied to chemoprevention of cancer.

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