Ciprofloxacin Mediated Cell Growth Inhibition, S/G_2-M Cell Cycle Arrest, and Apoptosis in a Human Transitional Cell Carcinoma of the Bladder Cell Line

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ABSTRACT

The second most prevalent urological malignancy in middle aged and elderly men is bladder cancer, with 90% of the cases being transitional cell carcinomas. The success of current systemic and intravesical therapeutic agents, such as cisplatin, thiotepa, Adriamycin, mitomycin C, and bacillus Calmette-Guérin, is limited with recurrence rates reduced to 17–44%. In addition, most of these agents require instrumentation of the urinary tract and are delivered at a significant cost and potential morbidity to the patient. Fluroquinolone antibiotics such as ciprofloxacin, which can be administered p.o., may have a profound effect in bladder cancer management. This is primarily based on limited *in vitro* studies on tumor cells derived from transitional cell carcinoma of the bladder that revealed a dose- and time-dependent inhibition of cell growth by ciprofloxacin at concentrations that are easily attainable in the urine of patients. However, the mechanism(s) by which ciprofloxacin elicits its biological effects on bladder cancer cells is not well documented. Our experimental data confirm previous studies showing the *in vitro* cell growth inhibition of the transitional cell carcinoma of the bladder cell line HTB9 and further showed the induction of cell cycle arrest at the S/G_2-M checkpoints. In addition, we found down-regulation of cyclin B, cyclin E, and dephosphorylation of cdk2 in ciprofloxacin-treated bladder tumor cells. There was also an up-regulation of Bax, which altered the Bax:Bcl-2 ratio, which may be responsible for mitochondrial depolarization reported to be involved prior to the induction of apoptosis. The cyclin-dependent kinase inhibitor $p21^{WAF1}$ level was found to be decreased within 12 h of ciprofloxacin treatment and disappeared completely when HTB9 cells were treated with 200 μg/ml ciprofloxacin for 24 h. The down-regulation of $p21^{WAF1}$ closely correlated with poly(ADP-ribose) polymerase cleavage and CPP32 activation. Recent studies revealed that $p21^{WAF1}$ protects cells from apoptosis by arresting them in G_1 and further binds to pro-caspase-3, preventing its activation and thus, inhibiting the apoptotic cascade. Hence, the down-regulation of $p21^{WAF1}$, together with the alterations in Bax and cdk2 as observed in our studies, may define a novel mechanism by which ciprofloxacin inhibits tumor cell growth and induces apoptotic cell death. The results of our current studies provide strong experimental evidence for the use of ciprofloxacin as a potential preventive and/or therapeutic agent for the management of transitional cell carcinoma of the bladder.

INTRODUCTION

Bladder cancer is the second most prevalent malignancy of the genitourinary tract in American men and the fourth most common cancer in terms of incidence (1, 2). An estimated 50,500 new cases are diagnosed annually, and 90% of these are transitional cell carcinoma (3). The rate of tumor recurrence of bladder cancer is as high as 66% of patients within 5 years of diagnosis and ~88% for those surviving 10 years (3, 4). At the time of diagnosis, 80% of the cancers are superficial (T_a, T_{is}, and T_1), and tumor progression occurs in approximately 15 and 50% of patients diagnosed with T_a and T_1 disease, respectively (4, 5). This recurrence rate may be attributable to the growth of a new cancer at remote sites or implantation and subsequent proliferation of cells released into the bladder at the time of endoscopic removal of the primary tumor (6). Tumor recurrences felt to be attributable to implantation of viable tumor cells released at the time of TURBT[2] are validated by the differences in sites of recurrences as compared to the primary tumor (6).

Patients with superficial bladder cancers with a significant risk of progression or recurrence are treated with TURBT, followed by prophylactic treatment with systemic administration of cisplatin, and treatment with intravesical agents such as Adriamycin, mitomycin C, thiotepa, and most recently, *bacillus Calmette-Guérin* (7). These agents have varying degrees of efficacy, with recurrence rates reduced to approximately 17–44% when compared with controls (7). These treatments may have side effects that are largely drug specific, including thrombocytopenia in 3–31% of the patients and leukopenia in 8–54% of the patients treated with thiotepa, genital rash because of mitomycin C in 6% of the patients, and drug-related bladder

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[2] The abbreviations used are: TURBT, transurethral resection of the bladder tumor; cdk, cyclin-dependent kinase; 7-AAD, 7-amino actinomycin D; PARP, poly(ADP-ribose) polymerase.

contracture in 16% of patients treated with doxorubicin (7, 8). Side effects from *bacillus Calmette-Guérin* therapy, the most common intravesical agent, include high fever, granulomatous prostatitis, pneumonitis, and hepatitis (9). Because of these side effects and the unacceptable recurrence rate after TURBT, alternative treatment modalities are needed to improve the disease-free interval for bladder cancer, as well as overall survival.

Fluroquinolone antibiotics, ciprofloxacin and ofloxacin, are relatively nontoxic antibiotics that can be administered p.o. and are found to be highly concentrated in the urine, suggesting that the bladder epithelium is significantly exposed to these antibiotics. Recently, they have been shown to have growth-inhibitory effects against human transitional cell carcinoma of the bladder cell lines, TCCSUP, T24 and J82 *in vitro* (10). However, the molecular mechanism(s) by which these agents show antitumor activity has not been elucidated. Fluroquinolones are inhibitors of prokaryotic DNA gyrase, a DNA topoisomerase (11, 12). Topoisomerase enzymes are essential for DNA packaging, transcription, and replication and for chromosomal separation during mitosis. Thus, their inhibition results in cytostasis and cell death (12). An exponentially higher level can be achieved in urine than in serum with increasing oral intake of ciprofloxacin. Thus, other tissues are protected from the potentially cytotoxic concentration of ciprofloxacin, but the malignant urothelial cells are critically exposed to induce irreversible cell death.

A retrospective European clinical study reviewed the clinical records of patients with superficial bladder cancer who had received a fluroquinolone antibiotic (perfloxacin) and those who had received cefotetan prior to a TURBT. The patients in the perfloxacin group had a lower tumor recurrence and prolonged disease-free interval ($P < 0.001$; Ref. 13). These *in vivo* data strongly suggest the antineoplastic activity of fluroquinolones against transitional cell carcinoma of the bladder.

On the basis of limited *in vitro* and *in vivo* data documenting the potential biological effect of ciprofloxacin and because of the lack of molecular studies, elucidating the molecular mechanism by which ciprofloxacin elicits its biological influence on bladder cancer cells, we investigated the effects of ciprofloxacin on a human transitional cell carcinoma of the bladder cell line, HTB9. In this report, we show that ciprofloxacin has a significant cell growth-inhibitory activity, which was observed with concomitant cell cycle arrest at the S/G_2-M checkpoints. Furthermore, ciprofloxacin was found to be an effective agent in the down-regulation of cyclin B, cyclin E, cdk2, and $p21^{WAF1}$. In addition, we also found that ciprofloxacin is an effective agent in the up-regulation of Bax, suggesting the possible molecular mechanism by which it induces apoptosis. Collectively, our results provide important molecular information, for the first time, to our knowledge that may explain the inhibition of cell growth and ultimate triggering of a cellular cascade by which ciprofloxacin may cause cellular demise of bladder cancer cells.

MATERIALS AND METHODS

Cell Proliferation Assay. The human bladder cancer cell line HTB9 was obtained from American Type Culture Collection (Rockville, MD). This transitional cell carcinoma of the bladder was cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Inc., Rockville, MD). Cells (2×10^5) were cultured in six-well culture plates for 24 h before use in the experiment. Culture medium was replaced with fresh medium containing the appropriate concentration of ciprofloxacin ranging from 50 to 400 μg/ml, and fresh medium with drug was added every 24 h. Cells were collected by trypsinization and counted in triplicate with trypan blue exclusion using a hemocytometer, and the cell growth curve was plotted using the PRIZM software program.

Cell Cycle Analysis. HTB9 cells were seeded at a density of 6×10^5 in 100-mm culture dishes and grown to 50% confluence. Subsequently, the cells were cultured in serum-free medium for 24 h and then treated with 200–300 μg/ml of ciprofloxacin for 24–72 h in complete medium. The cells were harvested by trypsinization, centrifuged at 2000 rpm for 5 min, washed in PBS, and resuspended in cold 70% ethanol. The cells were then subjected to flow cytometric analysis on FACStar Plus (Becton Dickinson, San Francisco, CA) after propidium iodide staining.

Protein Extraction and Western Blot Analysis. HTB9 cells were plated and cultured in complete medium and allowed to attach for 24 h, followed by the addition of 200–300 μg/ml of ciprofloxacin. The incubation was continued for 24, 48, and 72 h, respectively. Control cells were maintained in regular medium. Cells were harvested by scraping the cells from culture dishes with a scraper and collected by centrifugation. Cells were resuspended in 125 mM Tris-HCl buffer, sonicated with 10–20% output, and lysed using an equal volume of 8% SDS to make a final concentration of 4% SDS in the sample. Cell extracts were boiled for 10 min, chilled on ice, and centrifuged at 2000 rpm for 5 min before collecting the supernatant. The protein content of the samples was quantitated using the BCA protein assay kit (Pierce, Rockford, IL). Fifty μg of proteins were subjected to 14% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Each membrane was blocked with 10% dry milk prior to incubation with antibodies to $p21^{WAF1}$ (1:2000 dilution; Upstate, Lake Placid, NY), Bax (1:7500 dilution; Trevigen, Gaithersburg, MD), Bcl2 (1:1000 dilution; Dako, Carpinteria, CA), cyclin B (1 μg/ml; Neomarkers, Fremont, CA), cyclin E (1 μg/ml; Neomarkers), cdk2 (1:300 dilution; Neomarkers), or β-actin (1:2000 dilution; Sigma Chemical Co., St. Louis, MO), washed with TBST (Tris buffered saline, Tween 20), and incubated with secondary antibodies conjugated with peroxidase. The signal was detected using the chemiluminescent detection system (Pierce).

Northern Blot Analysis of $p21^{WAF1}$. To detect the $p21^{WAF1}$ levels at the transcriptional level, 10^6 cells were plated in 100-mm^2 dishes. Controls cells were maintained in complete media, but the treated cells were maintained in media with 200 μg/ml of the drug for 4, 8, 12, and 24 h, respectively. The RNA was extracted, and equal amounts were denatured at 65°C for 10 min and electrophoresed through a 1.4% agarose/2.2 M formaldehyde gel. The RNA separated on the gel was then blotted to a Gene Screen membrane by capillary transfer in 24 mM sodium phosphate buffer. The RNA on this membrane was fixed by exposure to UV light and subjected to prehybridization solution overnight at 68°C. Nick-translated ^{32}P-labeled $p21^{WAF1}$ cDNA

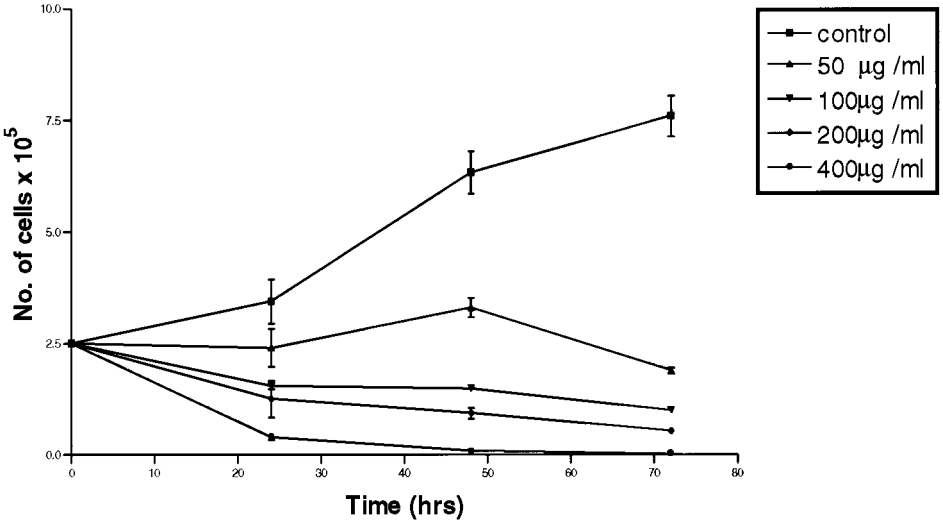

Fig. 1 Cell growth inhibition by ciprofloxacin. HTB9 cells were treated with 0–400 μg/ml ciprofloxacin, harvested by trypsinization, and counted. The number of living cells was plotted *versus* incubation time. The plot is an average of triplicate points for each treatment and representative of three independent experiments; *bars*, SD.

probe was added to the prehybridization solution and incubated overnight at 68°C. The membrane was then washed twice in 2X SSC, 1% SDS at 68°C for 5 min, and then three times in 0.1X SSC, 1% SDS at 68°C for 30 min. Autoradiographic analysis of the blot was carried out by exposing the membrane to Kodak X-OMAT X-ray film at −80°C with an intensifying screen.

Densitomeric Analysis. Autoradiograms of the Western blots were scanned with the Gel Doc 1000 image scanner (Bio-Rad, Hercules, CA) that was linked to a Macintosh computer. The bidimensional absorbances of $p21^{WAF1}$, Bcl-2, Bax, cyclin B, cyclin E, cdk2, and actin proteins, as well as $p21^{WAF1}$ mRNA and 28S rRNA on the films, was quantified and analyzed with the Molecular Analyst software program (Bio-Rad, Hercules, CA). The ratios of $p21^{WAF1}$:actin, Bax:Bcl-2, cyclin B:actin, cyclin E:actin, nonphosphorylated cdk2:actin, phosphorylated cdk2:actin, and $p21^{WAF1}$ mRNA/28S rRNA were calculated with standardizing the ratios of each control to the unit value.

Determination of Apoptotic Cell Death: 7-AAD Staining and Flow Cytometric Analysis. Cells were treated with ciprofloxacin for 24, 48, and 72 h, respectively. Control cells were kept in complete media without the drug. 7-AAD staining was carried out as described previously (14). Briefly, 7-AAD (Calbiochem-Novabiochem, La Jolla, CA) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/ml. A total of 100 μl of 7-AAD solution was added to 10^6 cells suspended in 1 ml of PBS and mixed well. Cells were stained in the dark for 20 min at 4°C and pelleted by centrifugation. The cells were resuspended in 500 μl of PBS/1% BSA solution. Unstained cells were used as a negative control, and for positive control, heat-killed cells were stained with 7-AAD. Samples were analyzed on a FACScan (Becton Dickinson, San Francisco, CA) within 30 min. Data on 20,000 cells were acquired and processed using Lysis II software (Becton Dickinson). Scatterograms were generated by combining forward light scatter with 7-AAD fluorescence, and regions were drawn around clear-cut populations having negative, dim, and bright fluorescence. The frequency of cells with low, medium, and high 7-AAD fluorescence was assessed.

Analysis of PARP Cleavage. Control cells and cells treated with 300 μg/ml ciprofloxacin for 12, 24, 48, and 72 h, respectively, were lysed in lysis buffer [10 mM Tris-HCl (pH 7.1), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 5 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100]. The lysates were kept on ice for 30 min and vigorously vortexed before centrifugation at 12,500 × *g* for 20 min. Fifty μg of the total protein were resolved through 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was incubated with primary monoclonal antihuman PARP antibody (1:5000; Biomol, Plymouth Meeting, PA), washed with TBST, and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescence detection system (Pierce).

Analysis of CPP32 (Caspase 3). Control cells and cells treated with ciprofloxacin for 24, 48, and 72 h, respectively, were lysed in lysis buffer [10 mM Tris-HCl (pH 7.1), 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, and 1% Triton X-100]. The lysates were kept on ice for 30 min and centrifuged at 12,500 × *g* for 20 min. Fifty μg of total protein were resolved through 14% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was incubated with primary monoclonal antihuman CPP32 antibody (1:200; Santa Cruz biotechnology, Santa Cruz, CA), washed with TBST, and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

RESULTS

Effect of Ciprofloxacin on Cell Proliferation. The treatment of HTB9 cells for 24–72 h with 50–400 μg/ml ciprofloxacin resulted in a dose-dependent decrease in cell proliferation (Fig. 1). In addition to cell growth inhibition, we also

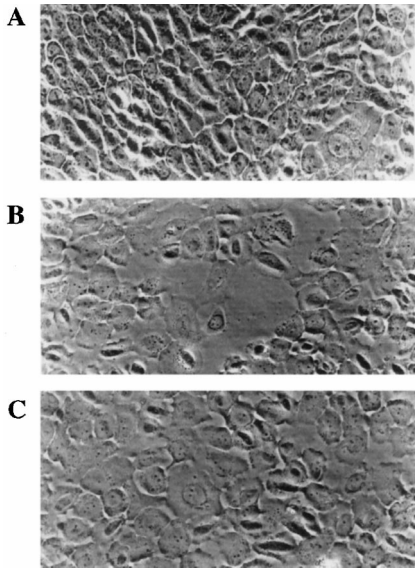

Fig. 2 Morphological alteration of ciprofloxacin-treated HTB9 cells. *A*, control (untreated) HTB9 cells. Treatment with 200 μg/ml ciprofloxacin for 3 days (*B*) and treatment with 200 μg/ml ciprofloxacin for 24 h, followed by reculturing in drug-free media for 48 h (*C*) are shown. ×200.

observed significant morphological changes that are presented in Fig. 2. The untreated control cells (Fig. 2*A*) did not show any morphological changes, whereas cells treated with ciprofloxacin showed altered cell morphology with cell blebbing, an early feature of apoptotic processes, and the cells were also found to be detaching from the culture plates. This effect was observed with 200 μg/ml of ciprofloxacin treatment for 72 h (Fig. 2*B*) and was found to be irreversible, as demonstrated in Fig. 2*C*, where cells treated with 200 μg/ml for 24 h were recultured in drug-free media for an additional 48 h. The data clearly document the antiproliferative activity of ciprofloxacin in HTB9 bladder tumor cells, and moreover, these morphological changes suggest that ciprofloxacin may also induce apoptotic cell death. To determine the potential cell cycle effect of ciprofloxacin, we investigated the distribution of cells in different phases of the cell cycle after ciprofloxacin treatment.

Ciprofloxacin Induces S/G_2-M Cell Cycle Arrest in HTB9 Cells. When cells were treated with 300 μg/ml ciprofloxacin for 72 and 96 h, we found a significant number of cells that were arrested at the S and G_2-M phases of the cell cycle. The result of a typical experiment is shown in Fig. 3, and the data are summarized in Table 1. In control cultures (Fig. 3), 55 and 66% of cells were in G_0-G_1 phase, 29 and 25% were in S phase, and 16 and 9% were in G_2-M phase at 72 and 96 h, respectively. However, in ciprofloxacin-treated cells, the number of cells in S phase was increased to 39 and 35% at 72 and 96 h, respectively. The relative number of cells in G_2-M phase was also increased to 38 and 43% after 72 and 96 h of treatment, respectively (Table 1). These data provide strong evidence for cell cycle arrest induced by ciprofloxacin and, in turn, the inhibition of cell growth. However, the reduced cell growth could also be attributable to the apoptotic cell death in addition to cell growth inhibition. Hence, we investigated whether ciprofloxacin could induce apoptotic cell death in bladder cancer cells.

Ciprofloxacin Induces Apoptosis. Ciprofloxacin was found to induce apoptotic cell death in the HTB9 cells in a dose- and time-dependent manner. Apoptosis was observed at 12 h, as indicated by the degradation of PARP and activation of CPP32 (caspase 3). Proteolytic processing of specific target proteins such as PARP has been shown to occur in cells exposed to a number of apoptotic stimuli (15–18). Western blot analysis of the cleavage of PARP showed a decrease in the full-size M_r 116,000 fragment and an increase in the M_r 85,000 cleaved fragment within 12 h after the bladder tumor cells were treated with 300 μg/ml of ciprofloxacin (Fig. 4*a*). Western blot analysis of CPP32 activation also showed that the CPP32 protein was cleaved to yield a M_r 17,000 fragment after 12 h (Fig. 4*b*). Activation of CPP32 triggers the activation of the interleukin converting enzyme cascade to initiate apoptotic cell death (16, 18). Furthermore, our studies with flow cytometric analysis of cells stained with 7-AAD also showed increased apoptosis at 48 h. Twenty-three and 31% of cells were found to be undergoing apoptotic cell death when treated with 200 and 300 μg/ml of ciprofloxacin, respectively, compared with 13% in control cells (Fig. 5). Collectively, these results provide strong evidence that apoptotic cell death is induced by ciprofloxacin in HTB9 bladder tumor cells. However, further studies are needed to determine the molecular mechanism by which ciprofloxacin induces apoptotic cell death in bladder cancer cells.

Ciprofloxacin Effects on the Expression of Bax and Bcl-2. The protein expression levels of Bcl-2 and Bax in cells treated with 200–300 μg/ml of ciprofloxacin for 24–72 h was studied by Western blot analysis. There was no effect on the level of Bcl-2 expression in treated cells. In contrast, the constitutive levels of Bax were altered in the treated cells. The expression level of Bax was up-regulated in bladder tumor cells treated with 200–300 μg/ml of ciprofloxacin after 24 h (Fig. 6). The optical densitometric analysis of Bax and Bcl-2 was done as described in "Materials and Methods." The data show the increase in Bax compared with Bcl-2 and was found to be dose dependent. The up-regulation of Bax was not transient because the level of expression was found to remain elevated after treatment for 72 h. The ratio of Bax over Bcl-2 was greater than 2-fold in favor of Bax, suggesting that this altered ratio could contribute to the apoptotic cell death observed in ciprofloxacin-treated cells. However, it is important to note that the translocation of Bax into mitochondria in the absence of Bax overexpression may also be sufficient for the induction of apoptotic processes. To further delineate the molecular mechanism of cell growth inhibition and apoptosis, we also investigated the protein expression of cell cycle, cell growth, and other apoptosis-related proteins in HTB9 cells treated with ciprofloxacin.

Modulation in the Expression of Cyclin B, Cyclin E, and cdk2 in Ciprofloxacin-treated Cells. Cyclin B associates with cdc2 and regulates transition through the G_2-M checkpoint of the cell cycle (19, 20). Cyclin E also associates with cdk2 to form kinase complexes that are active in late G_1 and early S phase (21). cdk2 is most active in the S and G_2 phases and has been implicated mainly in the control of the S-phase progression (21). Ciprofloxacin-treated HTB9 cells were found

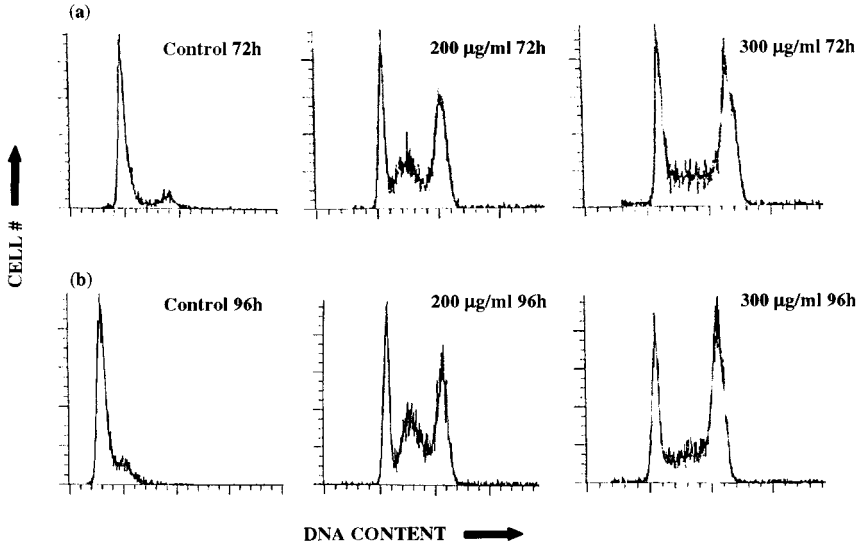

Fig. 3 Cell cycle arrest at the S/G_2-M phase of the cell cycle. HTB9 untreated control and treated with 200 and 400 μg/ml of ciprofloxacin for 3 (*a*) and 4 days (*b*), respectively. Cells were harvested as indicated in "Materials and Methods," and their DNA content was studied by FACscan analysis.

Table 1 Ciprofloxacin-induced cell cycle progression arrest at S/G_2-M in HTB9 cells

	HTB9 cells	
Cell cycle phase	Control cells (%)	300 μg/ml ciprofloxacin cells (%)
Day 3		
G_0-G_1	55	23
S	29	39
G_2-M	16	38
Day 4		
G_0-G_1	66	22
S	25	35
G_2-M	9	43

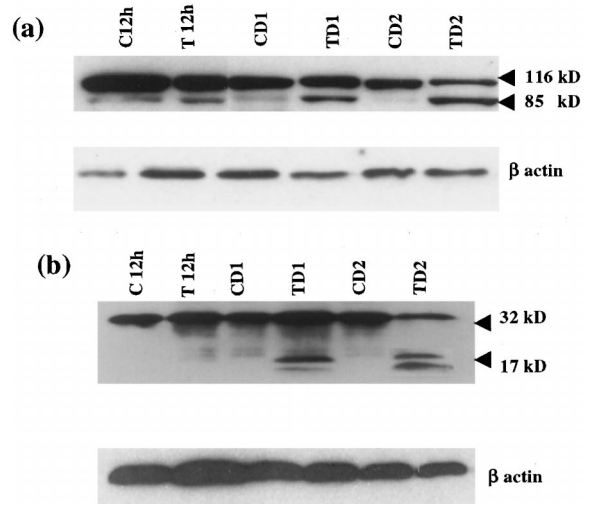

Fig. 4 Activation of apoptotic cell death induced by ciprofloxacin. Western blot analysis of PARP cleavage (*a*), where *C12h, CD1,* and *CD2* represent control cells at 12, 24, and 48 h of culture, respectively, and *T12h, TD1,* and *TD2* represent cells treated with 200 μg/ml ciprofloxacin for 12, 24, and 48 h, respectively. Western blot analysis of CPP32 activation (*b*), where *C12h, CD1,* and *CD2* represent untreated control cells at 12, 24, and 48 h of culture, respectively, and *T12h, TD1,* and *TD2* represent cells treated with 200 μg/ml ciprofloxacin for 12, 24, and 48 h, respectively.

to be arrested in the S/G_2-M phase of the cell cycle, suggesting modulation of cyclin/cdk complexes, which are important for regulating cell cycle progression (19–21). Western blot analysis revealed down-regulation of cyclin B and cyclin E at 48 h after treatment with 200–300 μg/ml of ciprofloxacin (Figs. 7 and 8). Immunoblot analysis of cdk2 revealed two distinct bands at M_r 33,000 and 32,000, which correspond to the phosphorylated and nonphosphorylated forms of the cdk2, respectively. After treatment with ciprofloxacin, there was a decrease in the M_r 32,000 phosphorylated active form of cdk2, with a corresponding increase in the nonphosphorylated cdk2 (Fig. 9). These results provide molecular clues to the cell growth and cell cycle arrest induced by ciprofloxacin in bladder cancer cells.

Effect of Ciprofloxacin on the Expression of $p21^{WAF1}$. The cdk inhibitory protein $p21^{WAF1}$ has been shown to be regulated by a growth factor signaling cascade and by p53 and may control cell cycle progression by changes in its level of expression and association with other proteins (22, 23). $p21^{WAF1}$ also plays a role as either the inducer or inhibitor of apoptosis (24–27). Proteolytic degradation of important cellular proteins, such as $p21^{WAF1}$, has been shown to be associated with apoptosis (28). The influence of ciprofloxacin-induced apoptotic cell death and cell cycle arrest on $p21^{WAF1}$ expression was examined in HTB9 cells. As shown in Fig. 10*A*, ciprofloxacin decreased the levels of $p21^{WAF1}$ protein at 12 h, which closely correlated with the time of appearance of the M_r 85,000 cleavage product of PARP. Moreover, the level of $p21^{WAF1}$ protein was not detectable by Western blot analysis when cells were treated with ciprofloxacin for 24 h, and that this disappearance in the levels of $p21^{WAF1}$ was closely correlated with the induction of apoptosis. To demonstrate whether the effect of ciprofloxacin was at the level of transcription, translation, or post-

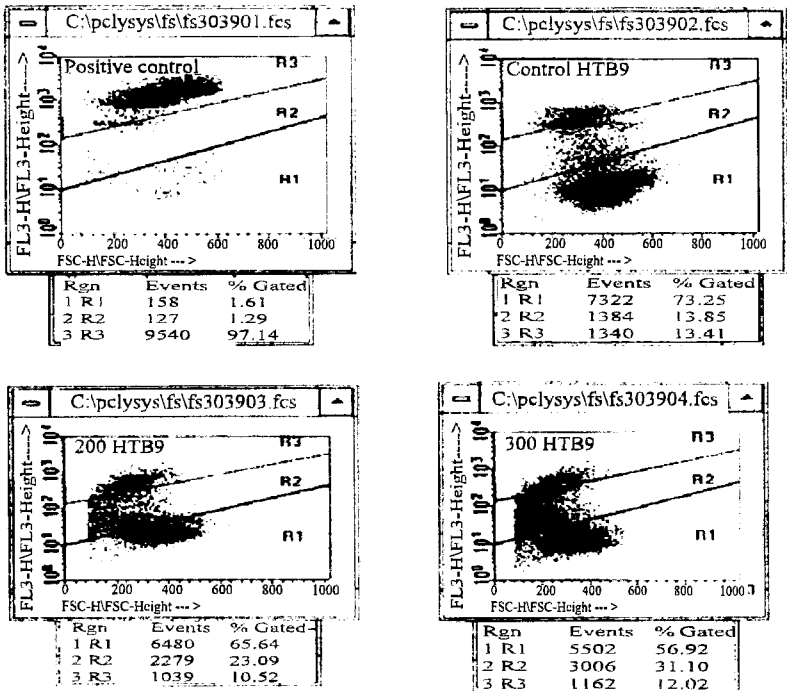

Fig. 5 Scatterograms of 7-AAD-stained cells. Scatterogram of positive control for apoptosis, where the cells were boiled (*left top panel*) and control cells that were cultured in drug-free medium for 48 h (*right top panel*) and ciprofloxacin-treated cells with 200 μg/ml (*left bottom panel*) and 300 μg/ml (*right bottom panel*) for 48 h, respectively. *R1*, live cells; *R2*, apoptotic cells; *R3*, dead cells.

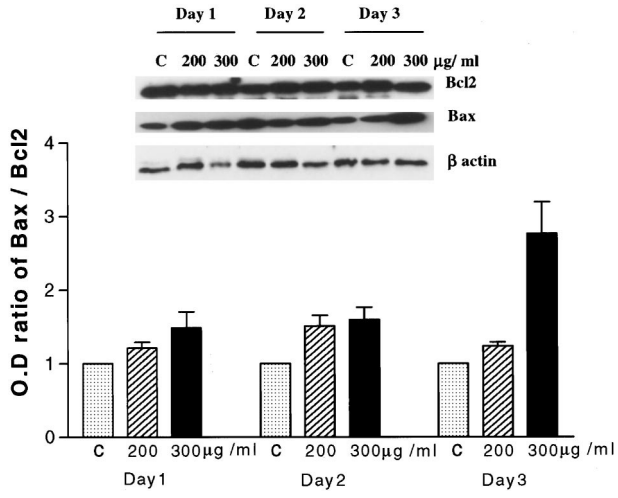

Fig. 6 Western blot analysis of Bax and Bcl-2 and densitometric analysis of the Bax:Bcl-2 ratio of HTB9 cells, where *C* represents untreated control cells, and ciprofloxacin-treated cells with 200 and 300 μg/ml for 24, 48, and 72 h, respectively. *Bars*, SD.

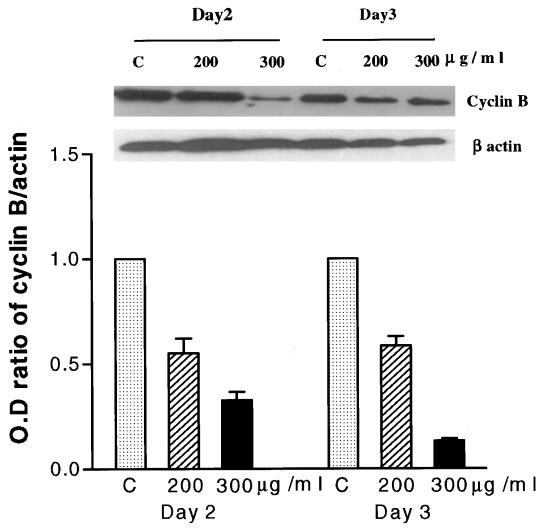

Fig. 7 Western blot and densitometric analysis of cyclin B in HTB9 cells, where *C* represents control cells, and ciprofloxacin-treated cells with 200 and 300 μg/ml for 48 and 72 h, respectively. *Bars*, SD.

translational, we investigated the levels of $p21^{WAF1}$ mRNA by Northern blot analysis. Ciprofloxacin treatment did not alter the level of $p21^{WAF1}$ mRNA (Fig. 10*B*) over a 24-h period, suggesting that the disappearance of $p21^{WAF1}$ must be posttranscriptional, which will require further in depth investigation in the future. However, the disappearance of $p21^{WAF1}$ may be attributable to the degradation of $p21^{WAF1}$ that may be caused by the activation of caspases which, in turn, induces apoptosis, as previously observed (28).

DISCUSSION

The antitumor activity of fluroquinolone antibiotics has only been investigated recently. There are few reports documenting the antiproliferative effect of quinolone antibiotics such as ofloxacin, levofloxacin, perfloxacin, and ciprofloxacin (29–31). A significant growth inhibition has been documented in a variety of human tumor cells, such as human leukemic cells, osteoblast-like MG-63 human osteosarcoma cells, and transitional cell carcinoma of the bladder (29–31). Our investigation

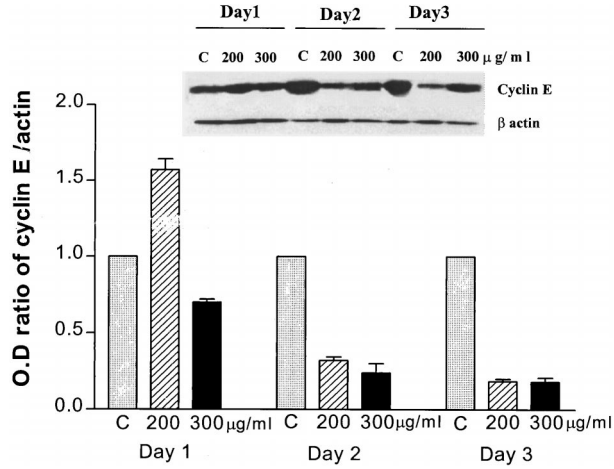

Fig. 8 Western blot and densitometric analysis of cyclin E in HTB9 cells, where *C* represents untreated control cells, and ciprofloxacin-treated cells with 200 μg/ml and 300 μg/ml for 24, 48, and 72 h, respectively. *Bars,* SD.

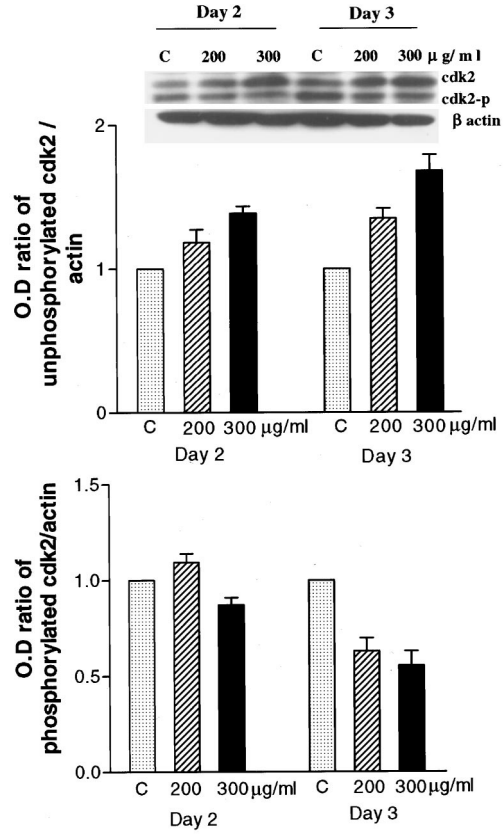

Fig. 9 Western blot and densitometric analysis of cdk2 in HTB9 cells, where *C* represents control cells, and ciprofloxacin-treated cells with 200 and 300 μg/ml for 48 and 72 h, respectively. *Bars,* SD.

also revealed an *in vitro* antiproliferative effect of ciprofloxacin on human transitional cell carcinoma of the bladder cell line, HTB9, in a dose-dependent manner. The growth inhibition ranged from 60 to 100% with 50–400 μg/ml of the drug, respectively, over a time course of 24–72 h. Cells treated with ciprofloxacin became rounded, detached from adjacent cells, and showed membrane blebbing, a typical feature prior to the initiation of apoptotic processes. The effect of ciprofloxacin at the morphological level was found to be irreversible, further suggesting that the cells were programmed to die when treated with ciprofloxacin. The flow cytometric analysis of cells treated with ciprofloxacin showed that the cells were arrested in S/G_2-M phases of the cell cycle. The induced cell cycle arrest was observed even at 96 h after treatment with 200–300 μg/ml of the drug, suggesting modulation of key cell cycle regulatory genes, which may be partly responsible for the cell cycle arrest at S/G_2-M transition in ciprofloxacin-treated bladder cancer cells.

In the present study, we further evaluated whether the overall growth inhibition induced by ciprofloxacin could also be attributed to apoptotic cell death in the bladder tumor cells. PARP is a common death substrate for activated enzymes of the caspase family. CPP32 is a key member of the family of caspases, which are the central component of the apoptotic machinery during apoptotic cell death. As shown in Fig. 4*b*, activation of CPP32 after ciprofloxacin treatment of the bladder tumor cells was confirmed by Western blot analysis, and the activation of CPP32 was closely correlated with the proteolytic cleavage of PARP. The 7-AAD staining analysis detected the altered cell membrane permeability in the apoptotic cells by the regulation of entry of the dye, which fluoresces red in the FL3 channel of the flow cytometer. The alteration of the Bax:Bcl2 ratio occurs significantly at 72 h, but there may be translocation of Bax to the mitochondria at 24 h without a significant up-regulation of the Bax protein to induce mitochondrial depolarization and subsequent activation of the interleukin converting enzyme cascade during initiation of apoptotic processes (23, 32). In many apoptotic scenarios, the mitochondrial inner transmembrane potential collapses with the release of cytochrome *c* into the cytosol, which results in activation of caspase 9, and also contributes to apoptosis by amplifying the effects of caspase 8 upon activation of downstream caspases (Fig. 11; Ref. 23). Taken together, our data provide convincing evidence for the antiproliferative activity and apoptosis-inducing effect of ciprofloxacin in bladder cancer cells.

CyclinB/cdc2-kinase accumulates during the G_2 phase and becomes activated at the G_2-M border by abrupt dephosphorylation of Thr-14 and Tyr-15 by the protein phosphatase cdc25c (19, 33). During mitosis, cyclin B/cdc2 kinase is inactivated by both degradation of cyclin B via a ubiquitin-dependent mechanism and dephosphorylation of Thr-161 (34). The down-regulation of cyclin B, as observed in cells treated with ciprofloxacin at 48 h, may partly explain the cell cycle arrest at G_2-M (38% of the cells were found to be arrested at G_2-M phase of the cell cycle (Table 1). Western blot analysis also revealed modulation of cyclin E and cdk2. The results of this study correlated well with the S/G_2-M cell cycle arrest induced in ciprofloxacin-treated bladder tumor cells. The inhibition of cdk2 phosphorylation was observed in ciprofloxacin-treated bladder cancer cells, as shown by the decrease in the rapidly migrating M_r 32,000

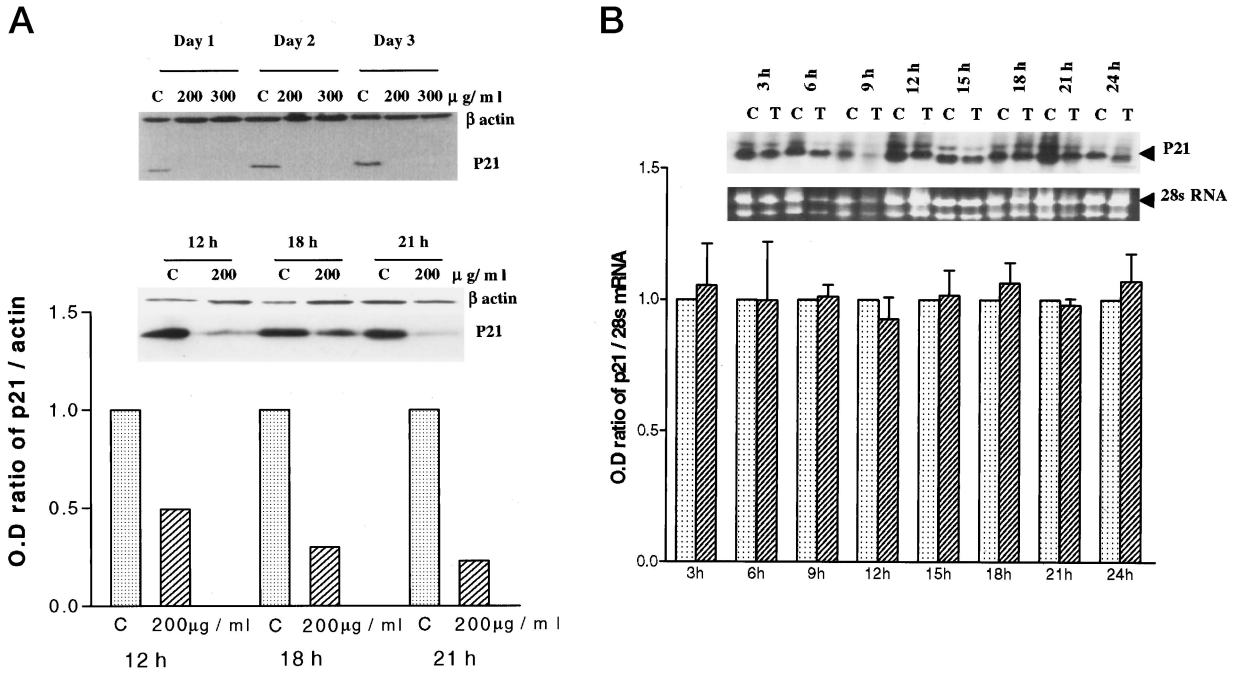

Fig. 10 Western (*A*) and Northern (*B*) blot analysis and densitometric presentation of p21^{WAF1} in HTB9 cells, where *C* and *T* represent control and ciprofloxacin treatment, respectively. *Bars*, SD.

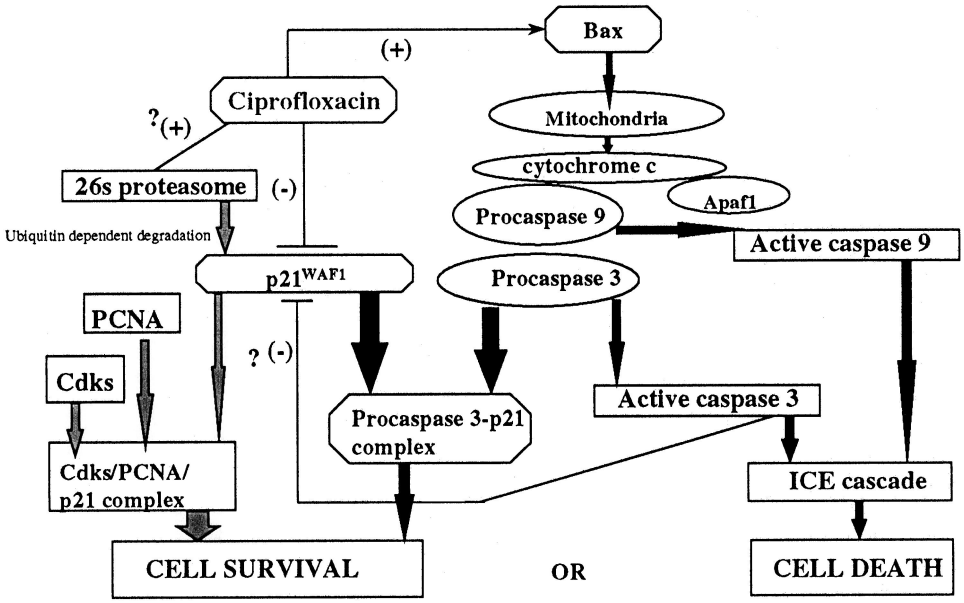

Fig. 11 Schematic diagram showing the potential biochemical pathway by which ciprofloxacin may inhibit cell growth and induce apoptosis in bladder cancer cells.

band, supporting its role in the inhibition of S-phase progression.

The cdk inhibitor p21^{WAF1} is a downstream effector of the p53-dependent cell growth arrest. It has been shown that p21^{WAF1} induces cell cycle arrest in G_1 and protects cancer cells from apoptosis induced by UV irradiation or RNA polymerase II blockage (35). In addition, inhibition of p21^{WAF1} has been shown to sensitize MCF-7 breast carcinoma cells and ME-180 osteosarcoma cells during tumor necrosis factor-induced apoptosis (36, 37). Previous studies also showed that p21^{WAF1} could protect colorectal cancer cells and human mesenchymal cells from apoptosis, and down-regulation of p21^{WAF1} resulted in cell death (38, 39). Our results show a decrease in p21^{WAF1} at the posttranscriptional level at 12 h, with a dramatic disappearance at 24 h, suggesting that the degradation of p21^{WAF1} may be mediated by caspase-dependent cleavage. Recently, it

was reported that caspase 3 could mediate the cleavage of $p21^{WAF1}$ at the site of DHVD1121L during the DNA damage-induced apoptosis (40, 41). The cleaved $p21^{WAF1}$ fragment can no longer arrest cells because it fails to bind the proliferating cell nuclear antigen and other effector molecules and, thus, loses its capability to localize to the nucleus, leading to acceleration of the chemotherapy-induced apoptotic process (40–42). Furthermore, it was shown that caspase 3 contains the $p21^{WAF1}$ binding domain in the NH_2 terminus, and formation of the $p21^{WAF1}$-procaspase complex protects it from the p3-site cleavage by serine proteinase, contributing to the apoptosis suppression machinery (39). Fig. 11 visualizes a schematic model of ciprofloxacin-induced cell death and also shows our hypothetical mode of action of ciprofloxacin in bladder cancer cells. In addition to protease-mediated cleavage of $p21^{WAF1}$, we also hypothesize that ciprofloxacin may mediate ubiquitination of $p21^{WAF1}$, followed by its degradation by the 26S proteasome complex pathway, because the ubiquitin degradation pathway has been found to be responsible for the degradation of several proteins like N-myc, c-myc, c-fos, p53, p27, and E1A, including $p21^{WAF1}$ (34, 43, 44). However, further in-depth studies are needed to demonstrate whether the down-regulation of $p21^{WAF1}$ is mediated through the ubiquitination pathway, or whether both the ubiquitination, as well as proteolytic pathways are involved in the degradation of $p21^{WAF1}$. The precise mechanism affecting the complete disappearance of $p21^{WAF1}$ to release procaspase-3 and, thereby, the initiation of the apoptotic cascade in ciprofloxacin-treated cells, remains to be firmly established.

Our data confirm results published previously on the *in vitro* inhibition of bladder tumor cell proliferation and, furthermore, shows that ciprofloxacin induces cell cycle arrest at the S/G_2-M checkpoints in transitional cell carcinoma of the bladder cell line, HTB9, at concentrations that can be easily attained in the urine of patients. The modulation of key cell cycle regulatory molecules, such as cyclin B, cyclin E, and cdk2, significantly contribute to the cell cycle progression arrest and cell growth inhibition induced by ciprofloxacin. Our data also provide strong evidence for the induction of apoptotic cell death, which may be attributable to the up-regulation of Bax that alters the Bax:Bcl-2 ratio in favor of proapoptosis. In addition, the dramatic decline of $p21^{WAF1}$ levels may also contribute to the ultimate demise of bladder cancer cells when exposed to ciprofloxacin. Taken together, our results provide molecular evidence for the first time to our knowledge on how ciprofloxacin may induce cell growth inhibition and apoptosis in bladder cancer cells. Hence, our results suggest that ciprofloxacin, which can be administered p.o., may ultimately prove useful as a potential preventive and/or therapeutic agent in transitional cell carcinoma of the bladder.

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