Theracurmin® efficiently inhibits the growth of human prostate and bladder cancer cells via induction of apoptotic cell death and cell cycle arrest

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Abstract. In the present study, we aimed to investigate the anticancer properties of Theracurmin®, a novel form of the yellow curry pigment curcumin, as well as explore the molecular mechanisms of the potential anticancer effects of Theracurmin® on human prostate cancer and bladder cancer cells *in vitro*. The proliferation of cancer cells was examined by using the Cell Counting Kit-8. The clonogenic growth potential was determined by clonogenic assay. Cell cycle distribution was evaluated by flow cytometry using propidium iodide staining. Western blot analysis was applied to explore the expression patterns of molecules associated with apoptotic cell death and cell cycle checkpoint. We noted that Theracurmin® and curcumin exhibited similar anticancer effects in both androgen-dependent and -independent human prostate cancer cells in a dose- and time-dependent manner. These agents reduced cell viability and clonogenic growth potential by inducing apoptosis and cell cycle disturbance in human prostate cancer cells. Theracurmin® and curcumin also exerted marked anticancer effects on human bladder cancer cells, even in cisplatin-resistant T24R2 cells, in a dose- and time-dependent manner. Moreover, Theracurmin® and curcumin treatment decreased cell viability and clonogenicity via induction of apoptotic cell death and cell cycle dysregulation in human bladder cancer cells. In conclusion, our study suggests that Theracurmin® has potential as an anticancer agent in complementary and alternative medicine for these urological cancers.

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Introduction

Recently, complementary and alternative medicine (CAM) has received great interest among cancer patients as an alternative therapeutic method (1). In fact, nearly 40% of cancer patients in the US may use at least one type of CAM approach (2). Physicians are particularly interested in using various foods as CAMs, and are trying to discover novel food-based CAMs owing to their pharmacologic safety and familiarity for use. Among many CAM agents, curcumin, a natural polyphenol derived from the rhizome of the plant *Curcuma longa*, has shown promise in the treatment of cancer patients (3). Curcumin has been shown to significantly inhibit the growth of many types of cancer cells by regulating various molecules associated with cancer cell proliferation (4). It also potentiates the anticancer effects of chemotherapeutic agents *in vitro* and *in vivo* (5-7).

However, the clinical efficacy of curcumin is still limited, which is most likely due to its low bioavailability (8). To address this drawback, Theracurmin® (nanocurcumin), a novel form of curcumin, was developed using a microparticle and surface-controlled drug delivery system (9). Theracurmin® shows improved bioavailability and water solubility compared to curcumin (9). Additionally, Theracurmin® improves the feasibility of *in vitro* testing and eventual *in vivo* administration (10,11). Despite these biological advantages, there have been no studies on the effects of Theracurmin® in urological cancer. Here, we investigated the anticancer properties of Theracurmin® on human prostate cancer and bladder cancer cells *in vitro* and compared them to those of curcumin. Moreover, we examined the relevant molecular mechanisms of the potential anticancer effects of Theracurmin® in these urological malignancies.

Materials and methods

Cell lines and reagents. Human prostate cancer cell lines (LNCaP, PC3, and DU145) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). We used RPMI-1640 medium (Welgene, Daegu, Korea) for LNCaP and PC3, and Dulbecco's modified Eagle's medium (DMEM;

Welgene) for DU145 cells as the basal culture medium. Human bladder cancer cell lines (T24, 253J, and HTB9) were also purchased from ATCC. To establish a cisplatin-resistant cell line (T24R2), we performed serial desensitization of T24 cells as previously described (12). The human bladder cancer cell lines were cultured in RPMI-1640 medium. All media were supplemented with 10% fetal bovine serum (Welgene), and 1% penicillin-streptomycin, and 1% non-essential amino acids (both from Invitrogen, Carlsbad, CA, USA). Curcumin (Sigma-Aldrich, St. Louis, MO, USA) and Theracurmin® (Handok Pharmaceuticals Co., Ltd., Korea) were prepared in dimethyl sulfoxide (DMSO) and diluted into the growth medium such that the final concentration of DMSO did not exceed 0.1% (v/v). Medium containing 0.1% DMSO was used as the negative control.

Cell proliferation assay. To examine the cell proliferation, we used the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). Cells ($2x10^3$ cells/well) were seeded onto 96-well plates and incubated for 24 h. Cells were treated with either curcumin or Theracurmin® for 24, 48, and 72 h, and then 10 μl of CCK-8 solution was added into the 96-well plates. After 4 h of incubation, the absorbance at 450 nm was determined using a plate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability results are reported as fold change compared to data derived from the first day of cell seeding.

Clonogenic assay. Cells ($1x10^3$ cells/well) were seeded onto 35-mm^2 dishes and treated with either curcumin or Theracurmin® for 48 h. To form visible colonies, the cells were cultured for an additional 14 day in either curcumin- or Theracurmin®-free culture condition. After fixation with 10% neutral-buffered formalin solution, the samples were stained with 0.1% crystal violet solution (both from Sigma-Aldrich). Finally, the samples were photographed, and the number of visible colonies comprising more than 50 individual cells was determined by using a SZX7 stereomicroscope (Olympus, Tokyo, Japan).

Flow cytometry for cell cycle analysis. Cells ($3x10^5$ cells/well) were plated on 60-mm^2 dishes and incubated with either curcumin or Theracurmin® for 48 h. The cells were fixed in 70% ethanol and reacted with RNase A (10 μg/ml) for 1 h at 37˚C. Then, cells were stained with propidium iodide solution (10 μg/ml) for 30 min at 4˚C in a dark room. The cell cycle distribution was analyzed using a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis. To extract total proteins, the cells were lysed with radio-immunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 1 mM phenylmethylsulfonyl fluoride]. After measuring protein concentrations of each sample using the BCA protein assay kit (Pierce, Rockford, IL, USA), samples were prepared with equal amounts of protein in 1X SDS buffer. Protein samples were electrophoresed on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After the samples were blocked with 5% (w/v) non-fat dry milk at room temperature for 1 h, the blots were incubated with primary antibodies overnight at 4˚C. Primary antibodies used in the present study were anti-cleaved caspase-3, -8, -9, poly(adenosine diphosphate-ribose) polymerase (PARP), cytochrome *c*, cyclin D1, cyclin E1, p-Akt, Akt, p-Erk, and Erk (1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min at room temperature. Finally, protein expression levels were detected using an Enhanced Chemiluminescence Western Blot Substrate kit (Pierce).

Statistical analysis. Three independent experiments were performed in triplicate, and the data are presented as mean ± standard error of the mean (SEM). The statistical analysis was conducted using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). We considered data with p-values <0.05 as significant, as determined by Turkey's multiple range tests.

Results

Theracurmin® significantly inhibits the proliferation of human prostate cancer cells. To examine the cytotoxic effects of Theracurmin® on human prostate cancer cells, we treated cells with this agent for 24, 48 and 72 h and examined the cell viability using the CCK-8 assay. Cell viability of the PC3, DU145 and LNCaP cells was significantly decreased in a dose- and time-dependent manner after Theracurmin® treatment (Fig. 1A). As shown in Fig. 1B, the cytotoxic effects of curcumin were similar to those of Theracurmin®. Moreover, we found that Theracurmin® markedly impaired the clonogenic proliferation of human prostate cancer cells in a dose-dependent manner (Fig. 1Ca and Da). We also confirmed that the effects of curcumin on clonogenic proliferation were similar to those of Theracurmin® (Fig. 1Cb and Db).

These results indicate that Theracurmin® and curcumin have similar anticancer effects on human prostate cancer cells in both androgen-dependent and -independent cells in a dose- and time-dependent manner.

Theracurmin® exerts anticancer effects by inducing apoptotic cell death and cell cycle disturbance in human prostate cancer cells. To further examine the molecular mechanisms of the anticancer activities of Theracurmin® in human prostate cancer cells, we investigated the expression patterns of molecules associated with apoptosis, as well as the cell cycle distribution patterns after Theracurmin® treatment. Western blot analysis showed that pro-apoptotic proteins (cleaved PARP, caspase-3, -8, and -9, cytochrome *c*, and Bad) were upregulated, whereas total PARP and anti-apoptotic molecule (Bcl-2) were downregulated after treatment with Theracurmin® in the PC3, DU145 and LNCaP cells, respectively (Fig. 2A). We also noted that the expression patterns of apoptosis-related proteins after curcumin treatment did not differ from those after Theracurmin® treatment (Fig. 2A). Additionally, Theracurmin® treatment resulted in cell cycle disturbances, such as G2/M arrest, remarkably in PC3 cells, but subtly in the DU145 and LNCaP cells (Fig. 2B). Consistent with these results, aberrant expression of regulatory molecules

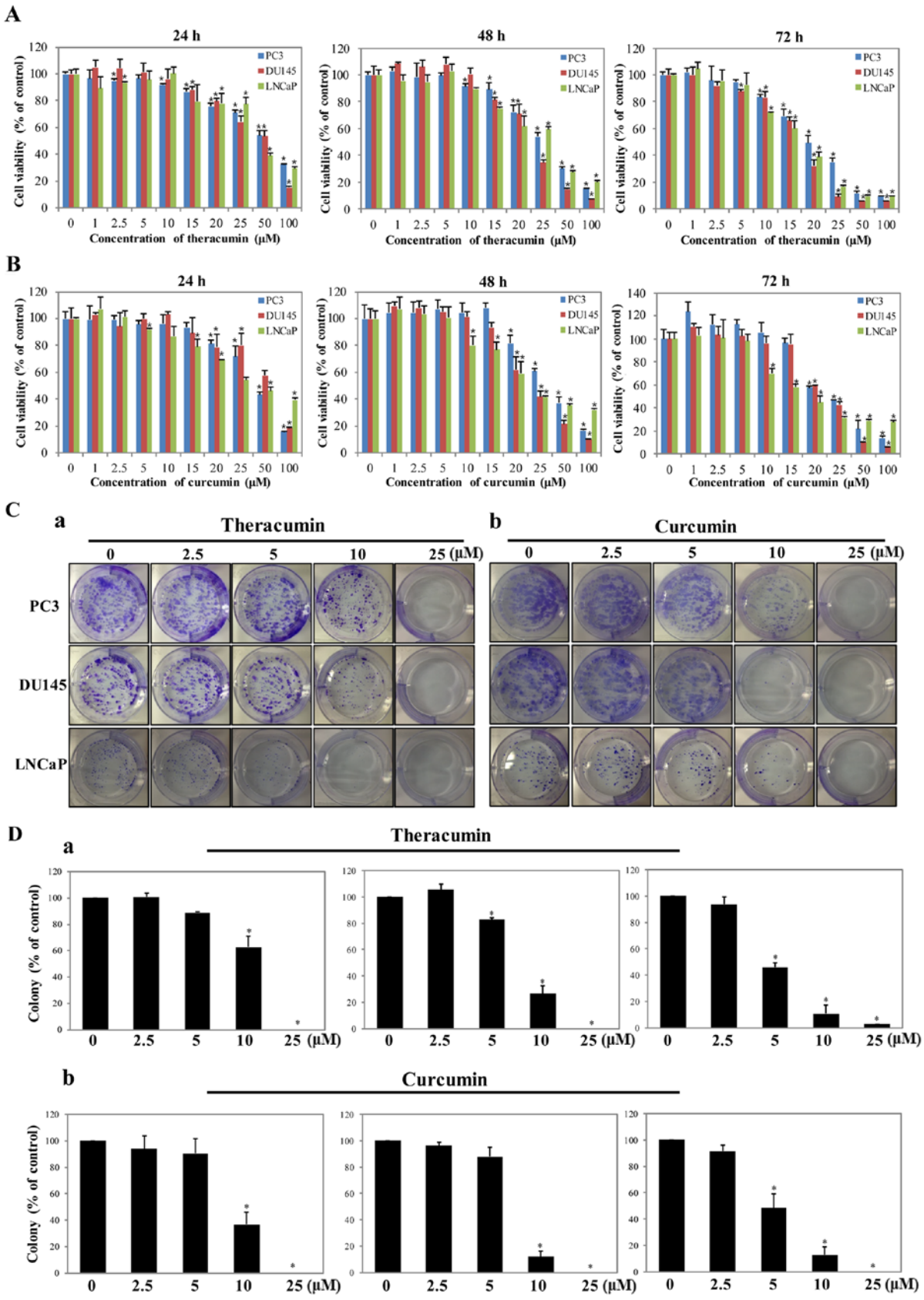

Figure 1. Anticancer effects of Theracurmin® and curcumin treatment in human prostate cancer cells. Cell viability assessments for determining cytotoxic effects of (A) Theracurmin® or (B) curcumin treatment in human prostate cancer cell lines (PC3, DU145, and LNCaP). The cells were treated with different concentrations of each agent, ranging from 1 to 100 μM, for 24, 48, and 72 h. Mock (0) was used as a negative control. The rates of cell viability are shown as the mean percentage of control ± standard error of the mean (n=3, *P<0.05). Clonogenic assay (C and D) for examining colony formation after 14 days of (a) Theracurmin® or (b) curcumin treatment in human prostate cancer cell lines (PC3, DU145 and LNCaP). Cells were treated with different concentrations of Theracurmin® (0, 2.5, 5, 10 and 25 μM) for 48 h. (C) Crystal violet staining was used for visualizing significant colonies consisting of more than 50 individual cells in (a) Theracurmin®- and (b) curcumin-treated groups. (D) The numbers of colonies are shown as representative bar graphs with the mean percentage of control ± standard error of the mean (n=3, *P<0.05).

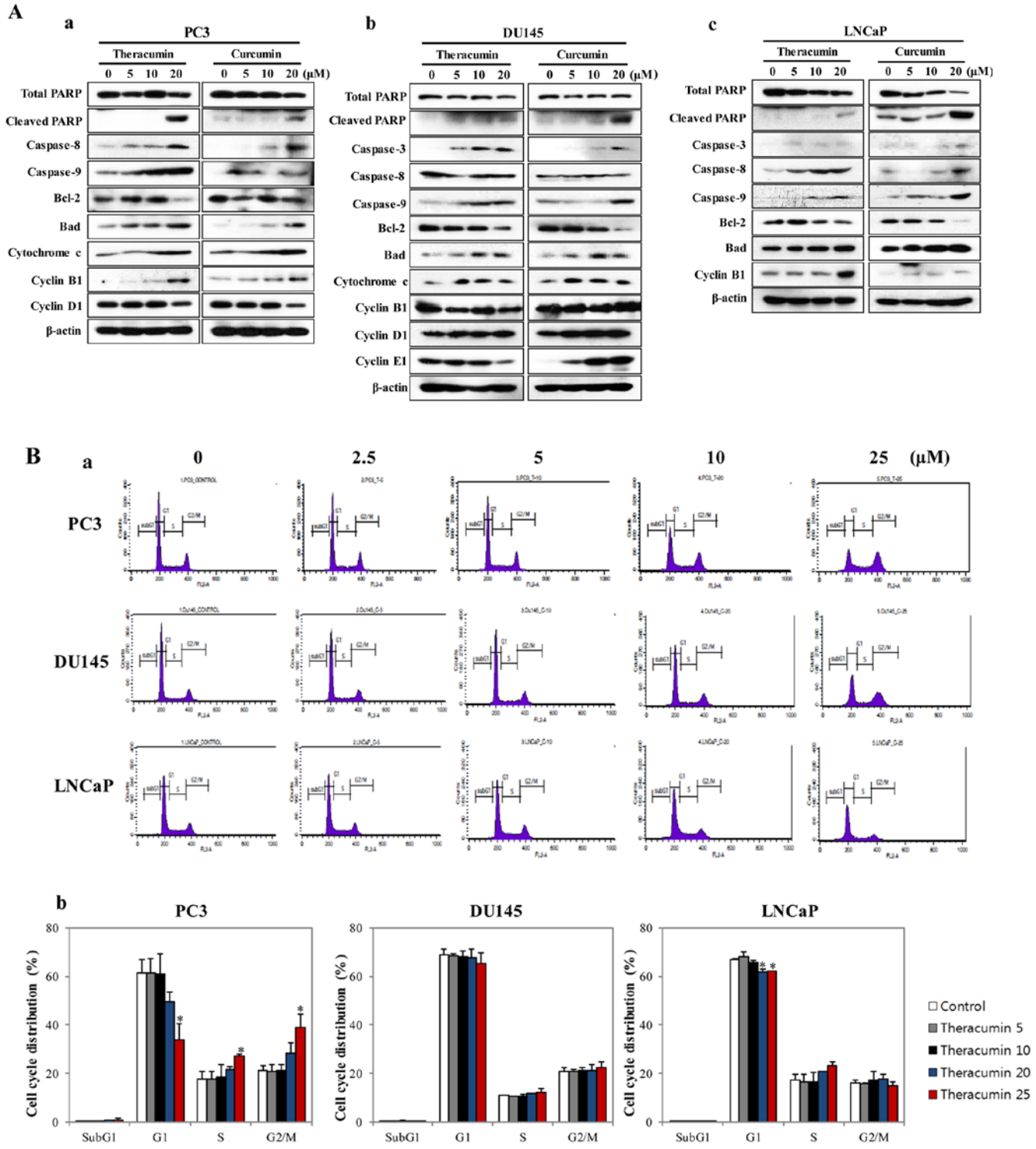

Figure 2. Theracurmin® and curcumin treatment induce apoptotic cell death and cell cycle dysregulation in human prostate cancer cells. (A) Western blot analysis for the expression of molecules associated with apoptosis (total PARP, cleaved PARP, caspase-3, caspase-8, caspase-9, Bcl-2, Bad, and cytochrome *c*) and cell cycle regulation (cyclin B1, cyclin D, and cyclin E) in human prostate cancer cells (a) PC3, (b) DU145, and (c) LNCaP after treatment with various concentrations (0, 5, 10 and 20 μM) of Theracurmin® and curcumin for 48 h. β-actin was used as a loading control. (B) Cell cycle analysis by flow cytometry with propidium iodide staining in human prostate cancer cells (PC3, DU145, and LNCaP) after treatment with various concentrations (0, 5, 10 and 20 μM) of Theracurmin® for 48 h: (a) the representative results and (b) the proportion of cells in cell cycle phases (G1, S, and G2/M) are presented as a percentage of the control.

at cell cycle checkpoints (cyclin B1, D1 and E1) was observed with higher doses of Theracurmin® (Fig. 2A). Similar to these findings, curcumin treatment also induced cell cycle dysregulation, particularly G2/M arrest (Fig. 2C), with irregular changes in the expression of relevant checkpoint molecules (Fig. 2A).

These findings suggest that Theracurmin® and curcumin treatment led to the reduction in cell viability and clonogenic

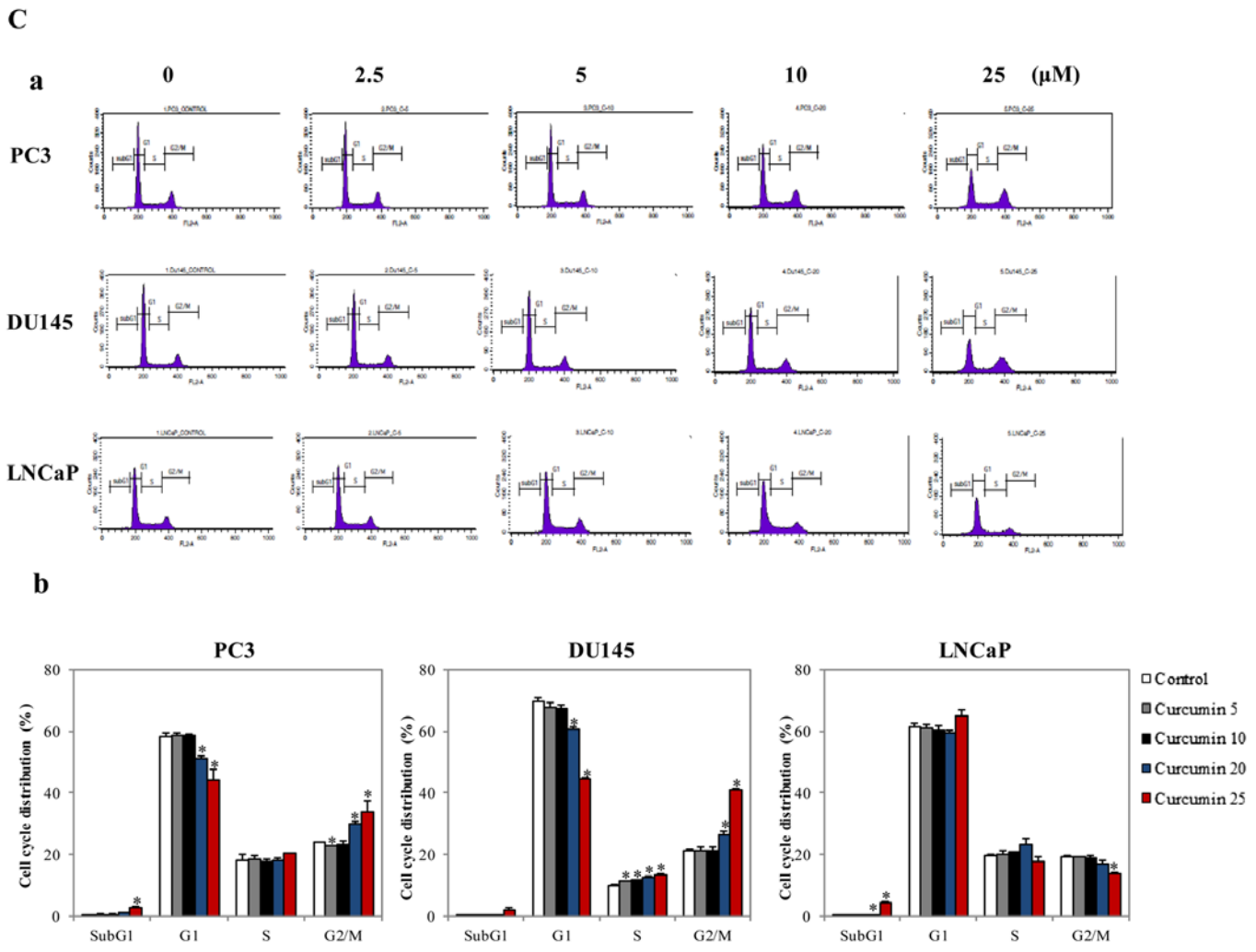

Figure 2. Continued. Theracurmin® and curcumin treatment induce apoptotic cell death and cell cycle dysregulation in human prostate cancer cells. (C) Cell cycle analysis by flow cytometry with propidium iodide staining in human prostate cancer cells (PC3, DU145, and LNCaP) after treatment with various concentrations (0, 5, 10 and 20 μM) of curcumin for 48 h: (a) the representative results and (b) the proportion of cells in cell cycle phases (G1, S, and G2/M) are presented as a percentage of the control.

potential via the induction of apoptotic cell death in human prostate cancer cells. Partly, cell cycle disturbance, such as G2/M arrest, played an important role in the cytotoxicity of Theracurmin® treatment in both androgen-dependent and -independent cells.

Theracurmin® treatment efficiently suppresses the growth of human bladder cancer cells. We next examined the cytotoxic effects of Theracurmin® on human bladder cancer cell lines (T24, 293J, and HTB9) as well as on the cisplatin-resistant cell line, T24R2. Similar to the results using prostate cancer cells, Theracurmin® treatment markedly reduced the viability of the bladder cancer cell lines in a dose- and time-dependent manner (Fig. 3A). Despite the subtle difference in responsiveness, the overall patterns of cytotoxic effects were similar between the cells treated with Theracurmin® and curcumin (Fig. 3B). Of note, these specific agents exerted a marked anticancer effect on the cisplatin-resistant T24R2 cells.

We revealed that Theracurmin® treatment also significantly diminished the clonogenic proliferation of human bladder cancer cells in a dose-dependent manner (Fig. 3Ca and Da). Although the anti-clonogenic effect of Theracurmin® was relatively lower than that observed with curcumin treatment (Fig. 3Cb and Db), our results indicate that Theracurmin® and curcumin have similar anticancer effects on human bladder cancer cells, even in cisplatin-resistant cells.

Anticancer effects of Theracurmin® are induced by apoptotic cell death and cell cycle dysregulation in human bladder cancer cells. To explore the molecular aspects of the anticancer effects of Theracurmin® in human bladder cancer cells, we examined the expression patterns of apoptosis- and cell cycle-regulating molecules after adding Theracurmin®. Similar to the findings in prostate cancer cells, the pro-apoptotic proteins (cleaved PARP, caspase-3, -8, and -9, cytochrome *c*, and Bad) were highly expressed, whereas total PARP and the expression of anti-apoptotic molecule Bcl-2 reduced in a dose-dependent manner after treatment with Theracurmin® (Fig. 4A). Additionally, cell cycle analysis revealed that this agent induced sub-G1 arrest, indicating apoptotic cell death (Fig. 4B). Theracumin also led

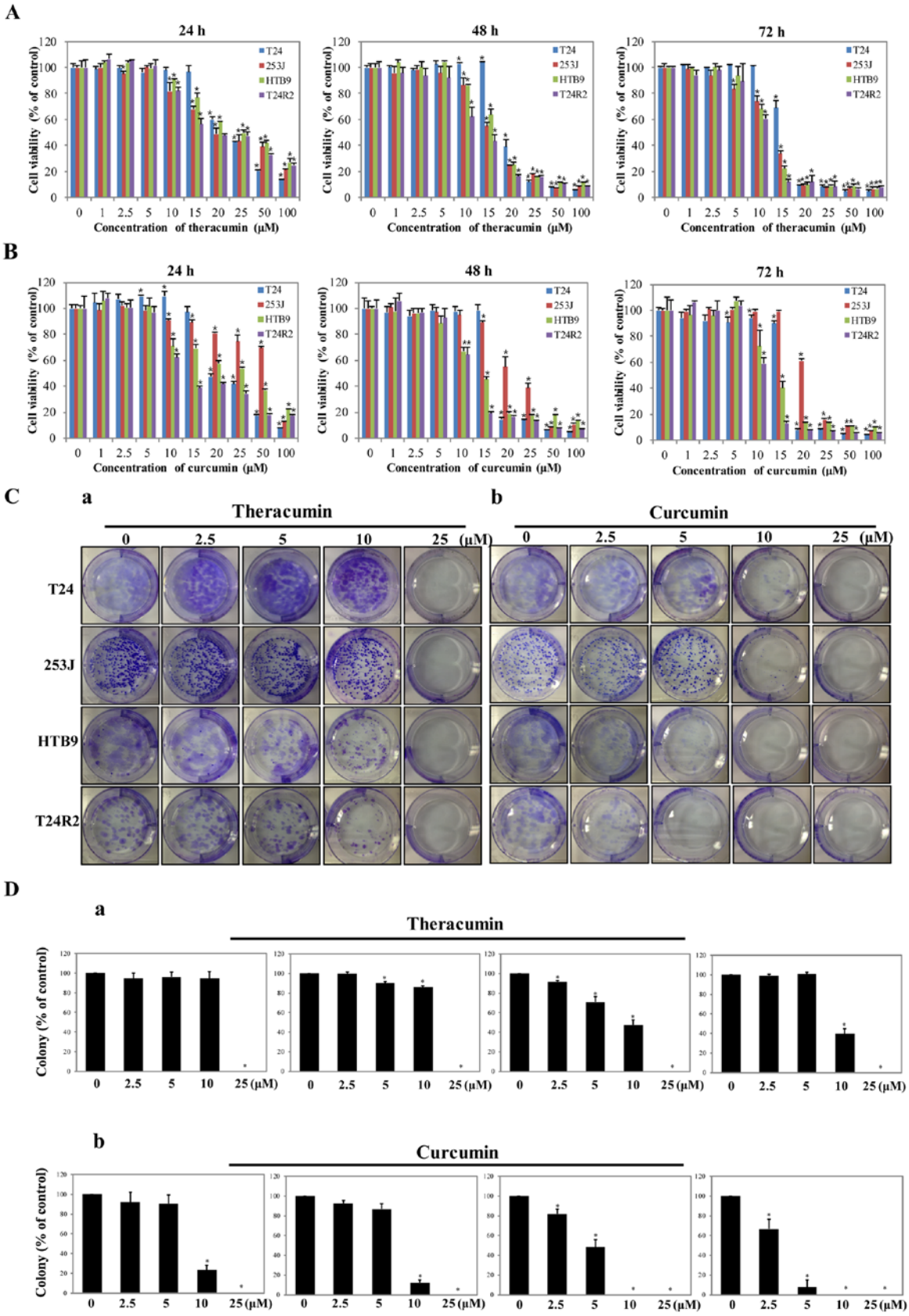

Figure 3. Anticancer effects of Theracurmin® and curcumin treatment in human bladder cancer cells. Cell viability assessments for determining the cytotoxic effects of (A) Theracurmin® or (B) curcumin treatment in human prostate cancer cell lines (T24, 253J, HTB9, and cisplatin-resistant T24R2). The cells were treated with different concentrations of each agent, ranging from 1 to 100 μM, for 24, 48 and 72 h. Mock (0) was used as a negative control. The rates of cell viability are shown as the mean percentage of control ± standard error of the mean (n=3, *P<0.05). (C and D) Clonogenic assay for examining the colony formation after 14 days of (a) Theracurmin® or (b) curcumin treatment in human prostate cancer cell lines (T24, 253J, HTB9, and cisplatin-resistant T24R2). Cells were treated with different concentrations of Theracurmin® (0, 2.5, 5, 10 and 25 μM) for 48 h. (C) Crystal violet staining was used for visualizing significant colonies consisting of more than 50 individual cells in (a) Theracurmin®- or (b) curcumin-treated groups. (D) The numbers of colonies are shown as representative bar graphs with the mean percentage of control ± standard error of the mean (n=3, *P<0.05).

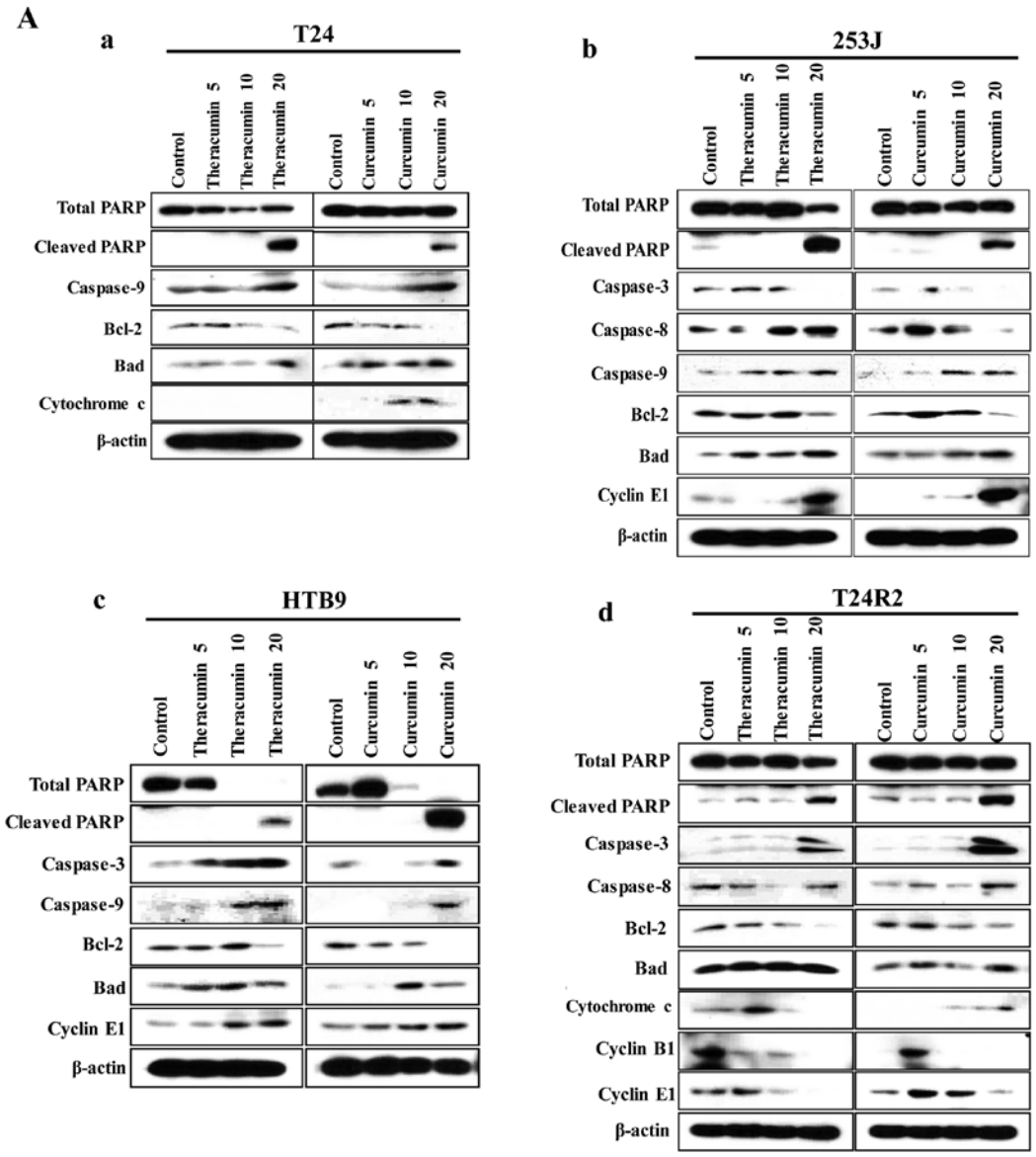

Figure 4. Theracurmin® and curcumin treatments induce apoptotic cell death and cell cycle dysregulation in human bladder cancer cells. (A) Western blot analysis for the expression of molecules associated with apoptosis (total PARP, cleaved PARP, caspase-3, caspase-8, caspase-9, Bcl-2, Bad, and cytochrome *c*) and cell cycle regulation (cyclin B1, cyclin D, cyclin E) in human bladder cancer cells (a) T24, (b) 253J, (c) HTB9, and (d) cisplatin-resistant T24R2 after treatments with various concentrations (0, 5, 10 and 20 μM) of Theracurmin® or curcumin for 48 h. β-actin was used as a loading control.

to cell cycle dysregulation, including S and/or G2/M phase arrest (Fig. 4B). In western blot analysis, the amounts of checkpoint molecules (cyclin B1 and cyclin D) reduced at higher doses of Theracurmin® in bladder cancer cells, even in cisplatin-resistant T24R2 cells (Fig. 4A). We also found that curcumin induced similar outcomes of apoptotic cell death and cell cycle dysregulation to those with Theracurmin® (Fig. 4A and C). Our findings indicate that Theracurmin® and curcumin have comparable anticancer effects by reducing cell viability through the induction of apoptotic cell death and cell cycle arrest in the various types of human bladder cancer cells.

Discussion

Prostate and bladder cancer are usually diagnosed in older men, who may be vulnerable to the side effects from first-line chemotherapeutic agents, such as docetaxel and cisplatin. In this context, natural compounds can be beneficial as an alternative therapeutic approach for treating these urological cancers (13). Many *in vitro* and *in vivo* studies suggest that curcumin, a yellow curry pigment, can significantly inhibit the growth, proliferation, invasive capabilities, and metastatic potential of prostate and bladder cancer cells (14-16).

Although many preclinical studies have shown curcumin to be a promising anticancer agent in urological cancer, its poor bioavailability is a major drawback to its clinical application for cancer patients (13). The limited oral bioavailability of curcumin may be attributed to its poor solubility in water and extensive systemic metabolism after oral intake. For example, a dose-escalation study carried out by Lao *et al* (17) demonstrated that no curcumin was detected in the blood of 24 individuals after oral administration of curcumin at

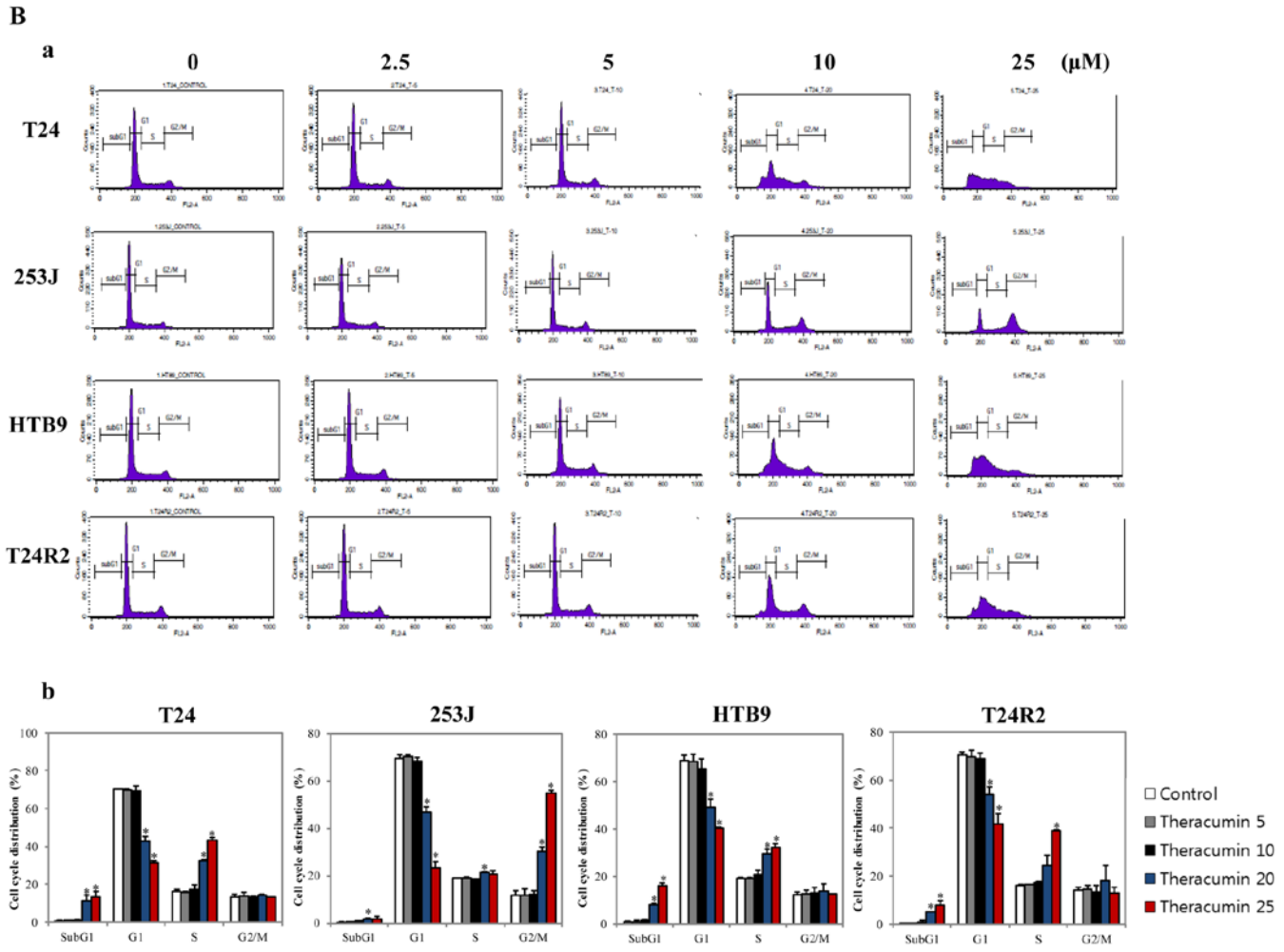

Figure 4. Continued. Theracurmin® treatment induces apoptotic cell death and cell cycle dysregulation in human bladder cancer cells. (B) Cell cycle analysis by flow cytometry with propidium iodide staining in human bladder cancer cells (T24, 253J, HTB9, and cisplatin-resistant T24R2) after treatment with various concentrations (0, 5, 10 and 20 μM) of Theracurmin®. (a) The representative results and (b) the proportion of cell in cell cycle phases (G1, S, and G2/M) are presented as a percentage of the control.

various doses ranging from 500 to 8,000 mg. To overcome this obstacle, an innovative form of curcumin as a nanoparticle colloidal dispersion (Theracurmin®) has been developed with higher stability and improved bioavailability (9). Several clinical trials have shown that Theracurmin® has no significant adverse effects in human subjects, indicating that this novel agent is well tolerated (9,18-22). Moreover, Theracurmin® significantly inhibited the proliferation of esophageal adenocarcinoma cells (OE33 and OE19), whereas it did not impair the proliferation of normal esophageal cells (HET-1A) in a recent study by Milano *et al* (21).

Despite these promising results showing Theracurmin® to be a safe and effective anticancer agent, no proof-of-concept studies have been carried out in urological cancer. In this study, we first revealed that Theracurmin® significantly reduced the cell viability in human prostate cancer and bladder cancer cells, particularly showing comparable anticancer efficacy with curcumin. Notably, the anticancer effects of Theracurmin® were exerted by inducing apoptotic cell death and cell cycle disturbance in these urological cancer cells. Similar to our findings, Kamat *et al* (5) revealed that curcumin suppressed the proliferation of bladder cancer cells (RT4V6 and KU-7) in a dose-dependent manner by inducing cell cycle arrest and potentiating the apoptotic effects of chemotherapeutic drugs. Park *et al* also showed that curcumin treatment induced growth inhibition of T24 bladder cancer cells driven by cell cycle disturbance, specifically G2/M phase arrest (22). In a study by Aggarwal *et al*, curcumin enhanced the anticancer effects of chemotherapeutic agents by degradation of cyclin E expression via upregulation of p21 and p27, resulting in G1 phase arrest in LNCaP prostate cancer cells (23).

Apoptosis and cell cycle arrest, such as in the S or G2/M phases, are regarded as the cellular response pathways following diverse stimuli, including DNA damage (24). In this regard, these distinct processes are intimately related and together play a crucial role in the responsiveness of cancer cells to anticancer agents (24). The complex interplay of various regulators is fundamental in processes involved in the progression of apoptotic cell death and the cell cycle. For apoptosis, there are two classifications of regulatory molecules: those that modulate mitochondrial function (e.g., Bcl-2, Bcl-xL, and Bax) and those that regulate the apoptotic cascades (e.g., caspase-3, -8 and -9) (25). For cell cycle regulation, there are two key classes of regulatory proteins, cyclins and cyclin-dependent

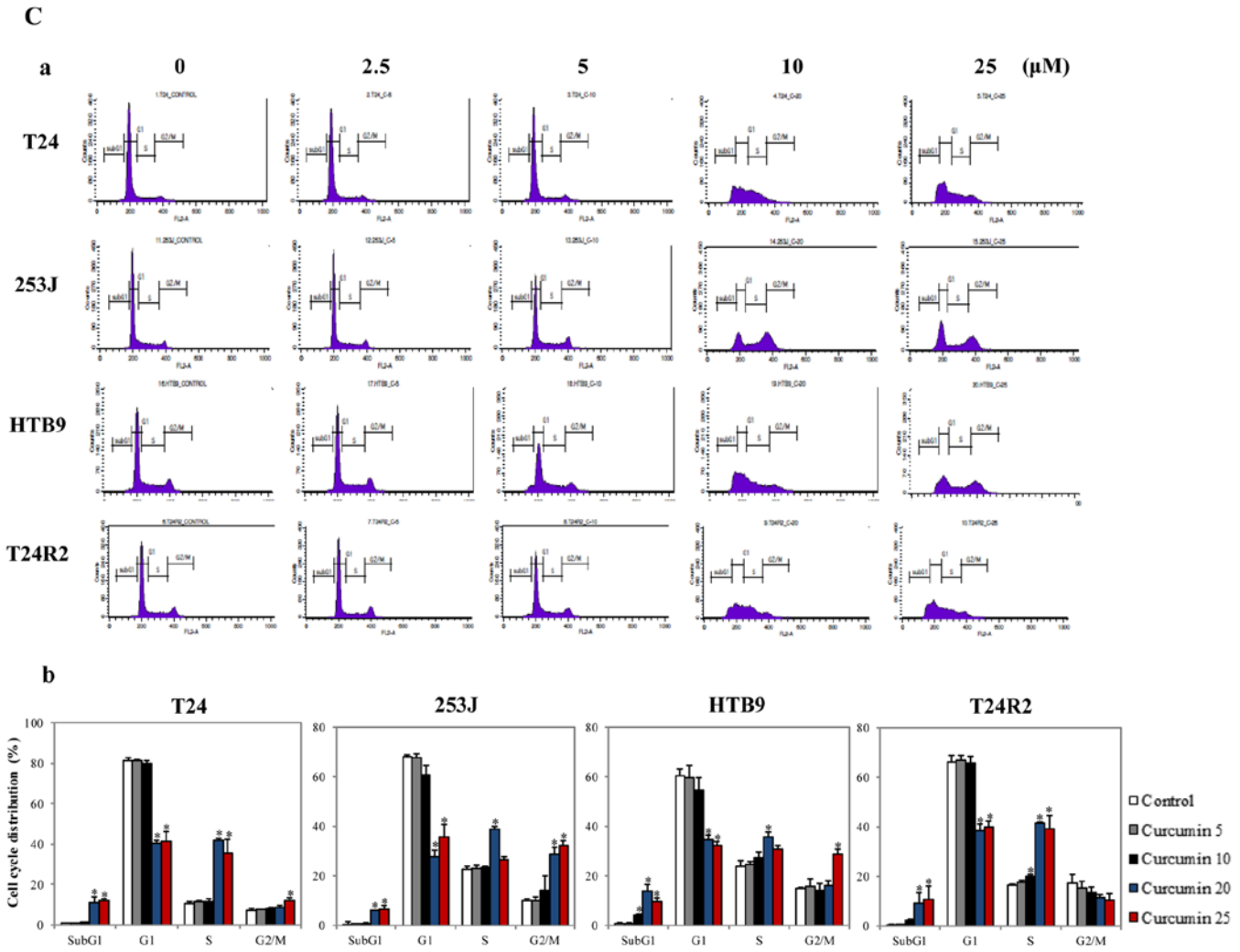

Figure 4. Continued. Curcumin treatment induces apoptotic cell death and cell cycle dysregulation in human bladder cancer cells. (C) Cell cycle analysis by flow cytometry with propidium iodide staining in human bladder cancer cells (T24, 253J, HTB9, and cisplatin-resistant T24R2) after treatment with various concentrations (0, 5, 10 and 20 μM) of curcumin for 48 h. (a) The representative results and (b) the proportion of cells in cell cycle phases (G1, S, and G2/M) are presented as a percentage of the control.

kinases (CDKs). For example, cyclin E and CDK2 play an important role in the progression of G1 through S phase, as well as cyclin B and CDK1 are key regulatory molecules for the G2/M phase (26).

Mechanistically, curcumin has been shown to modulate many molecular targets, including transcription factors, oncogenic growth factors, and inflammatory cytokines with relevant multiple signaling pathways, resulting in inhibition of cell proliferation, invasion and metastasis (27). The activity of NF-κB, which is constitutively activated in several malignancies, can be inhibited by curcumin treatment in several cancer cells. The expression of Wnt/β-catenin signaling pathway, frequently upregulated during initiation and progression of several tumors, can also be blocked by curcumin (28,29). For instance, curcumin reduces the accumulation of nuclear β-catenin and its target protein Tcf-4 in colon cancer cells, leading to growth arrest and apoptotic cell death (30). Curcumin can regulate other well-known oncogenic signaling pathways, such as STAT3 and PI3K/AKT, by suppressing their phosphorylation (31,32). Additionally, curcumin is a potent inhibitor of several pro-inflammatory cytokines, such as IL-1, IL-5, IL-8 and IL-12, involved in tumor angiogenesis and metastasis (33).

However, our study did not provide a more comprehensive data for the molecular mechanisms of the anticancer activities of Theracurmin® in human prostate and bladder cancer cells. Our data only demonstrated the phenotypic changes of human prostate cancer and bladder cancer cells after Theracurmin® and curcumin treatment, which is the major limitation of our study. In order to provide more concrete evidence, we should explore the relevant signaling pathways. More importantly, this study only provided *in vitro* results for the anticancer effects of Theracurmin® in both prostate cancer and bladder cancer cells. Thus, an *in vivo* efficacy study using xenograft models is required to consolidate our results and show that Theracurmin® treatment can be a useful CAM approach for both prostate cancer and bladder cancers.

In summary, we provides *in vitro* evidence that Theracurmin®, an innovative nano-form of curcumin, exhibits anticancer effects by inducing apoptotic cell death and cell

cycle dysregulation in human prostate and bladder cancer cells. Further mechanistic studies are required to fully appreciate the clinical potential of Theracurmin® as a promising alternative medicine agent in these urological cancers.

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