Activation of the Wingless (Wnt)/ß-catenin signaling pathway contributes to prostate tumorigenesis and metastasis. Depending on the stage of prostate cancer development, current drug therapies are of limited efficiency, so that prevention with natural compounds appears as an attractive strategy especially due to the slow progressive development of prostate cancer.

We report here that the chemopreventive agent curcumin from the rhizome of *Curcuma longa* was able to affect cell proliferation of androgen-dependent prostate cancer through the induction of cell cycle arrest in G2 and modulation of Wnt signaling. Curcumin decreases the level of Tcf-4, CBP and p300 proteins implicated in the Wnt transcriptional complex that leads to the decrease of ß-catenin/Tcf-4 transcriptional activity and of the expression of ß-catenin target genes (*cyclin D1* and *c-myc*). Subsequent cell death induction is linked to autophagy. Interestingly, in androgen-independent prostate cancer cells, curcumin does not affect Wnt/ß-catenin transcriptional activity. Altogether our results suggest that curcumin is an interesting chemopreventive agent for early stage prostate cancer.

**Introduction**

Deregulation of the Wingless (Wnt/ß-catenin) signaling pathway is well described to contribute to prostate cancer progression (1-4). The canonical Wnt signaling pathway is a complex process that regulates the ability of the multifunctional ß-catenin protein to activate the transcription of specific target genes involved in cell proliferation (e.g., *c-myc, cyclin D1*), cell adhesion (e.g., *E-cadherin*) and other signaling pathways [e.g., *collagenase-2 (Cox-2), matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF)*] (5).

In the absence of Wnt ligands, ß-catenin binds to a multi-protein degradation complex composed of Axin, glycogen synthase kinase-3ß (GSK-3ß), adenomatous polyposis coli (APC), protein phosphatase 2A (PP2A) and casein kinase 1 (CK1). ß-catenin is then phosphorylated by GSK-3ß and degraded by the proteasome. In contrast, binding of Wnt ligands to the Frizzled cell surface receptor and its low-density lipoprotein receptor-related protein 5 or 6 (LRP 5/6) co-receptor inhibits the assembly of the ß-catenin degradation complex and favours the translocation of ß-catenin to the nucleus where it dimerizes with the T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factor to activate the transcription of Wnt target genes (6).

Many natural compounds were reported to exhibit their chemopreventive potential through the modulation of the canonical Wnt signaling pathway in several cancer cell types (7-11). Curcumin (diferuloylmethane), from the roots of *Curcuma longa* and well characterized for its anti-carcinogenic, anti-proliferative, anti-inflammatory, anti-angiogenic and anti-oxidant properties (12,13) was also shown to affect the Wnt signaling especially in colon, gastric, intestinal and breast cancer cells (14-17). Studies evaluating the effect of curcumin on prostate cancer cell proliferation mainly focused on the impact of this natural compound on epidermal growth factor receptor (EGFR), on cyclins implicated in cell cycle and on phosphorylated isoforms 3-kinase (PI3)/Akt/mammalian target of rapamycin (mTOR) (18). Here we evaluate the effect of curcumin on prostate cancer cell proliferation mainly focused on the impact of this natural compound on epidermal growth factor receptor (EGFR), on cyclins implicated in cell cycle and on phosphorylated isoforms 3-kinase (PI3)/Akt/mammalian target of rapamycin (mTOR) (18). Here we evaluate the effect of curcumin on prostate cancer cell proliferation mainly focused on the impact of this natural compound on cell cycle and Wnt/ß-catenin in both androgen-dependent and independent prostate cancer cells, representative respectively of the early localized and late metastatic stages of prostate cancer development (19).

We report that curcumin essentially affects cell proliferation of androgen-dependent prostate cancer cells through the induction of cell cycle arrest in G2/M phase and through the...
decrease expression of β-catenin target genes due to the modulation of Wnt transcriptional activity mediated by the decrease of the level of expression of proteins implicated in the Wnt transcriptional complex. Interestingly, this natural compound induces cell death by autophagy in these androgen-dependent prostate cancer cells.

Materials and methods

Cell culture and chemicals. Androgen-sensitive (22rv1, LNCaP) and androgen-independent (DU145, PC-3) prostate cancer cells were cultured in RPM-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (Lonza) and 1% (v/v) antibiotic-antimycotic (Lonza) at 37°C, in a 5% CO₂, humidified atmosphere. Curcumin (Sigma, Bornem, Belgium) was dissolved in 100% DMSO (Sigma) at 20 mM. Subsequent dilutions were made in cell culture medium. Exponentially growing cells were subjected to curcumin treatment.

Cell viability. Prostate cancer cells in exponential growing phase were incubated with different concentrations (10, 20, 50, 75, 100 μM) of curcumin for 4 or 24 h. Cell viability was assessed by trypan-blue exclusion test.

Fluorescence microscopy. After incubation with 20 μM curcumin for 24 h, 22rv1 and DU145 cells were subjected to nuclear staining with 1 μg/ml Hoechst 33342 (Calbiochem, VWR International, Leuven, Belgium). Analysis of labeled cells was realized using an inverted Cell M microscope (Olympus, Aartselaar, Belgium) and the related Cell M software.

Preparation of total RNA. Nucleic acids were extracted from 10⁷ treated or untreated cell pellets with ‘Nucleospin RNA II’ kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer’s instructions. Dosage of RNA content was performed by using Nanodrop system and their quality was evaluated by absorbance ratio 260/280 nm of Warburg-Christian.

Real-time PCR. cDNA was synthetized by reverse transcription (RT) of 1 μg of total RNA in a final volume of 20 μl using oligo(dT) primers, dNTP mix, RNase out and Superscript II Reverse Transcriptase (Invitrogen, Tournai, Belgium). The resulting cDNA products were used for subsequent quantification by real-time PCR amplification with a 7300 Real-Time PCR System (Applied Biosystems, Halle, Belgium). Samples were amplified with different human gene specific primers (Eurogentec, Seraing, Belgium) presented in Table I. PCR was performed in a reaction mixture containing 1X Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.1 μM of each primer. Cycling conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles through a denaturing step at 95°C for 15 sec and an annealing-elongation step at 60°C for 2 min. The 7300 Sytem Sequence Detection Software Version 1.3.1.21 (Applied Biosystems) was used for the estimation of the Cₜ parameter (threshold cycle). The 2⁻ΔΔCₜ method was applied for the quantification and β-actin was used as housekeeping gene for the normalization (20).

Preparation of protein extracts. After treatment, 10⁷ cells were harvested and total, nuclear or cytoplasmic proteins were extracted as previously described (21). Protein content was determined for each sample using the Bradford assay (Bio-Rad protein Assay, Biorad, Nazareth, Belgium).

Western blot analysis. Proteins from total, cytoplasmic or nuclear extracts were subjected to SDS-PAGE and transferred onto a Hybond™-P membrane (GE Healthcare, Diegem, Belgium). Membranes were pre-hybridized overnight at 4°C in PBS 1X containing 0.1% (v/v) Tween-20 (PBS-T) and 5% milk. Hybridizations with primary antibodies, directed against β-catenin, Tcf-4 (Cell Signaling), PCNA, CBP, cyclin D1 (Santa Cruz), cyclin B1, phospho-histone H3, p300 (Millipore), c-myc (BD Pharmingen), survivin (R&D Systems) or β-actin (Sigma-Aldrich) used as a loading control, were then carried out in PBS-T containing 5% milk or 5% bovine serum albumin for 1 h at room temperature or overnight at 4°C. Membranes were then washed and probed with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Proteins of interest were visualized with Ecl Plus Western blotting Detection System (GE Healthcare) reagent using the Kodak Image Station (Analis, Suerlée, Belgium).

Transient transfection. Transient transfections of 22rv1 and PC-3 cells were performed by electroporation using the Bio-Rad gene Pulser. For each experiment, 3.75x10⁶ cells at a concentration of 1.5x10⁷ cells/ml were electroporated at 250 V and 975 μF in the presence of 5 μg of pTOPFlash or pFPFlash reporter gene construct harboring three copies of Tcf-4 binding site (wild-type or mutated respectively) upstream of the thymidine kinase minimal promoter and the open reading frame of luciferase gene (Upstate, Biognost, Heule, Belgium) and 5 μg ph-RG-tk Renilla plasmid, used as a control for transfection efficiency (Promega, Leiden, The Netherlands). Co-transfections were performed by addition of pbCAT expression vector coding for mutated β-catenin containing an activating S33Y mutation. After 48 h, transfected cells were treated or not with 10, 20 or 50 μM curcumin during 24 h. At the end of the treatment, 75 μl of 10⁶ cells/ml were placed in microtitration plates. Dual-Glo™ Luciferase Reagent (Promega) (75 μl) were added to the cells for 10 min at 22°C and then, 75 μl Dual-Glo Stop&Glo® Reagent (Promega) for 10-min incubation at 22°C. Luciferase and Renilla activities were measured on Orion microplate luminometer (Berthold Detection Systems). Results are expressed as a ratio of pTOPFlash/pFPflash luciferase activity normalized to Renilla activity.

Cell cycle. Cell cycle was analyzed according to standard procedures (22). Briefly, cells were fixed with an ethanolic solution in water (70% v/v) and DNA stained by treatment with propidium iodide (1 μg/ml; Becton-Dickinson Biosciences, Erembodegem, Belgium) and RNase A (100 μg/ml; Roche, Luxembourg) in PBS. Events were recorded statistically (10,000 events/sample) using CellQuest software associated with FACSCalibur (Becton-Dickinson Biosciences). Data were further analyzed by using WinMDI software (http://facs.scripps.edu/software.html).
Statistical analysis. Results from at least three independent experiments were analyzed for statistical significant differences using the Student’s t-test. They are expressed as the mean ± SD. p-values <0.05 (*) or 0.01 (**) were considered as statistically significant.

Results

Curcumin intracellular localization in prostate cancer cells. Fluorescent micrographs of 22rv1 and DU145 prostate cancer cells double stained with Hoechst 33342 and curcumin are presented in Fig. 1. The representative images of curcumin are shown in green (left panel), nuclei are visualized in blue (middle panel), and the merge of these two panels correspond to the right panel on which overlapped area should appear in yellow. In both cell lines, curcumin presents an intracellular fluorescence distribution in cytoplasmic compartments with no obvious localization inside the nucleus.

Effect of curcumin on prostate cancer cells viability. Cell viability assessed by trypan-blue exclusion test after curcumin treatment revealed that this natural compounds is cytotoxic...
in both androgen-dependent and -independent prostate cancer cells for concentration >75 μM. However, androgen sensitive prostate cancer cells are more sensitive to curcumin treatment (IC_{50} = 44 and 48 μM for 22rv1 and LNCaP, respectively) than their androgen-independent (IC_{50} = 115 and 170 μM for PC-3 and DU145 respectively) counterpart (Fig. 2).

Curcumin induces cell death by autophagy in 22rv1 cell. In order to understand whether the reduction of cell viability observed after curcumin treatment was due to apoptotic or autophagic cell death mechanisms, we performed Western blot analysis monitoring the cleavage of several caspases implicated in the extrinsic or intrinsic apoptotic cell death pathway but also LC3B linked to autophagy process (Fig. 3). In contrast to the positive control U937 cells treated with etoposide, curcumin does not induce the cleavage of pro-caspase-8, -9, -3, -7 and PARP but leads to the appearance of LC3B-II isomor, which amount is closely correlated with the number of autophagosomes, that serves as a good indicator of autophagy (23). Altogether these results support the idea that curcumin does not trigger cell death by apoptosis in our prostate cancer cell model, but is linked to the induction of autophagy.

Curcumin modulates 22rv1 cell cycle. The analysis by flow cytometry of propidium iodide stained cells revealed that curcumin is able to induce an arrest in G2/M transition phase of the cell cycle that fits with a decrease of the amount of cells in G1 phase after 24 h of 22rv1 cells treatment with 20 μM of curcumin (Fig. 4A). These modulations of cell cycle after curcumin treatment were confirmed by real-time PCR and Western blotting showing that curcumin decreases cyclin B1, PCNA (proliferating cell nuclear antigen) and phospho-histone H3 mRNA and protein level of expression (Fig. 4B and C). No significant increase of the percentage of 22rv1 cells was observed in sub-G1, reflecting normally cells dying by apoptosis (Fig. 4A). Here it represents <8% of total cell amount.

Inhibitory effect of curcumin on Wingless transcriptional activity and target genes. To determine whether androgen-dependent and -independent cells differ in their proliferative potential after curcumin treatment, we evaluated the effect of this natural compound on Wnt/β-catenin/Tcf-4 transcriptional activity. Activation of the Wnt pathway was monitored by co-transfection of Tcf-reporter plasmid pTOP-Flash (TOP) and pFOPFlash (FOP). These experiments revealed that curcumin is able to decrease the β-catenin/Tcf-4 transcriptional activity in androgen-dependent cells (22rv1), but not in androgen-independent (PC-3) prostate cancer cells. In fact, 20 μM of curcumin inhibits β-catenin/Tcf-4 signaling by 30% in 22rv1 cells and increases it by 50% in PC-3 cells.
The decrease of ß-catenin/Tcf-4 signaling transcriptional activity subsequently leads to a decrease of the expression of ß-catenin target genes such as c-myc, cyclin D1 and survivin (Fig. 6).

Figure 3. Curcumin induces cell death by autophagy in 22rv1 cells. Western blot analysis of the cleavage of pro-caspases -8,-9,-3,-7, Bcl-xL, cleavage of PARP (specific markers of cell death by apoptosis) and of the appearance of LC3B-II isoform (specific marker of autophagy). Results are representative of at least three independent experiments.

Figure 4. Curcumin modulates the proliferation of 22rv1 cells. 22rv1 androgen-dependent prostate cancer cells were treated with different concentration of curcumin for 24 h. (A) After harvesting, cells were stained with propidium iodide and cell cycle was analysed by flow cytometry. (B) The relative amount of PCNA and cyclin B1 mRNA was determined by real-time PCR analysis. Actin was used as internal control. A value of 1 was assigned to non-treated cells. (C) Total protein level of PCNA, cyclin B1 and phosphohistone H3 were analysed by Western blotting. Results are the mean ± SD of at least three independent experiments. *p<0.05 and **p<0.01 compared to non-treated cells, respectively.

(Fig. 5). The decrease of ß-catenin/Tcf-4 signaling transcriptional activity subsequently leads to a decrease of the expression of ß-catenin target genes such as c-myc, cyclin D1 and survivin (Fig. 6).
Effect of curcumin proteins implicated in the Wnt transcriptional complex. In order to determine the mechanism of action of curcumin on Wnt signaling in 22rv1 cells, we activated the Wnt-pathway by cotransfection of a β-catenin expression vector, containing an activating S33Y mutation (pbCAT), with pTOPFlash (TOP) and pFOPFlash (FOP) Tcf-reporter plasmids. We observed that even mutant β-catenin was able to activate β-catenin-dependent transcription (24), curcumin was unable to modulate this activation (Fig. 7A). This suggests that curcumin does not decrease Wnt transcriptional activity through an effect on β-catenin by itself. This hypothesis was confirmed by Western blot analysis showing that curcumin does not affect the level of expression of β-catenin protein (Fig. 7B). On the contrary, we report that curcumin decreases the expression of Tcf-4 and of its co-repressor CREB binding protein (CBP) and p300, three proteins involved in the Wnt transcriptional complex (Fig. 7B).

Discussion

On the one hand the aberrant activation of the canonical Wingless/β-catenin signaling pathway is a key element involved in prostate cancer development and progression (3,25), on the other hand, the anti-proliferative potential of curcumin was reported to be highly mediated by the modulation of cyclins, especially through the modulation of the Wnt transcriptional activity in colon, osteosarcoma and breast cancer cells (13,14,16,17,26,27). In the present study, we presented evidence that this natural chemopreventive compound has on effect on cell death and on cell proliferation-mediated by Wnt signaling in androgen-dependent but not in androgen-independent prostate cancer cell lines, and that the difference observed is not linked to a different intracellular localization of curcumin (Fig. 1).

In several cancer cell lines, including prostate cancer cells, curcumin was mainly reported to be an inducer of cell death by apoptosis through the intrinsic and extrinsic pathways (28-32). However, this natural compound was also shown to induce cytotoxicity, G2/M arrest and non-apoptotic/autophagic cell death in malignant glioma cell lines through the inhibition of Akt signaling pathway, the increase
of β-catenin expression level and the appearance of LC3II isoform (33-35). In our experimental approach, we pointed out that curcumin does not induce cell death by apoptosis in androgen-dependent 22rv1 cells (no cleavage of caspases nor PARP, <8% of cells in sub-G1) (Figs. 3 and 4A) but leads to cell death by autophagy as shown by the appearance of LC3B-II isoform and the decrease of Bcl-xL expression (Fig. 3). A link has been established between autophagic cell death and Bcl-2 anti-apoptotic proteins level of expression as it was reported that Bcl-xL binds and inhibits Beclin 1, an essential mediator of autophagy (36,37). Similarly, induction of specific features of apoptosis-independent cell death corresponding to autophagy was observed in oesophageal cancer cells after curcumin treatment (38). Autophagy was reported to be a back-up cell death mechanism when other cell deaths mechanisms failed and is now considered as a target for novel approaches in anticancer therapy (39).

Moreover, depending of cell lines tested, curcumin was shown to induce cell cycle arrest in G1 phase in glioma (40) and prostate cancer cells (41,42) or in G2/M phase in colon (17,31,43) and breast cancer cells (14). In our experimental approach, flow cytometry analysis pointed out that curcumin was able to induce cell cycle arrest in G2/M. The decrease of protein expression of cyclin B1, PCNA and of phospho-histone H3 specific mitosis marker suggested that this natural compound effectively induces an arrest in G2 phase of the cell cycle in 22rv1 cells (Fig. 4). Such an arrest in G2/M and concomitant decrease in cyclin B1 expression or decrease in PCNA expression were also observed in HCT-116 colon cancer cells (17), in BxPC-3 human pancreatic cancer cells (44), in human breast cancer cells (14,45) and in human bladder cancer T24 cells (46) after curcumin treatment. Jaiswal et al., suggested that such arrest in G2/M cell cycle phase in HCT-116 cells could result from an impairment of both Wnt signaling and cell-cell adhesion pathways.

This decrease of 22rv1 cell proliferation suggests that curcumin could exhibit an effect on the Wnt signaling pathway as it was reported that the suppression of this Wnt pathway inhibits prostate cancer cell proliferation (11). We then assessed the effectiveness of curcumin on prostate cancer cell proliferation and especially on the Wnt pathway.

We observed that curcumin is able to decrease the level of the Wnt/β-catenin/Tcf-4 transcriptional activity in androgen-dependent (22rv1) cells that leads to a decrease of the expression of β-catenin target genes (47,48) such as c-myc, cyclin D1 and survivin (Fig. 6), that are critical for tumor growth and survival, whereas no inhibitory effect was observed on Wnt transcriptional activity in androgen-independent (PC-3) cells. Survivin, a well-described member of the inhibitor of apoptosis (IAP), is an attractive curcumin target in cancer as it is differentially expressed in tumor vs. normal tissues (not expressed in normal prostate secretory epithelium but strongly expressed in prostate cancer cells). Survivin appears as an important regulator of the G2/M phase of the cell cycle and is normally required for maintaining cancer-cell viability and invasion by prostate cancer metastasis (49). Cyclin D1 is a known proto-oncogene that usually forms a complex with cyclin-dependent kinase (CDK), which activity is required for the transition form G1 → S (50) and which down-regulation by curcumin was already related to the suppression of cell proliferation in breast, prostate and squamous cell carcinoma (14,51). Such decrease of cyclin D1 observed after curcumin treatment (Fig. 6B) fits with previously published experiments performed in LNCaP androgen-dependent cells (51) and with the decrease of cell amount in G1 phase observed in Fig. 4A. By the same way, c-myc, another β-catenin target gene (48) was shown to be decreased by curcumin treatment in correlation with a G2/M cell cycle arrest, a decrease of β-catenin/ Tcf-4 transcriptional activity and of nuclear β-catenin and Tcf-4 expression in HCT-116 colon cancer cells (16,17) but never in prostate cancer cells.

Such inhibitory effect of curcumin on the Wnt transcriptional activity was thus already observed in human osteosarcoma cell lines (52), in colon (16,17) and breast cancer cells (14) but till now nothing was reported in the literature concerning the effect of curcumin on the Wnt signaling pathway in prostate cancer cells. In the case of breast cancer, low cytostatic dose of 20 μM curcumin was reported to induce an arrest in G2/M cell cycle phase, that was linked to the decrease of Wnt/β-catenin pathway components, such as nuclear disheveled and β-catenin proteins, but also the positively-regulated β-catenin targets, cyclin D1 and slug (14). Such curcumin-induced suppression of cell proliferation was correlated with down-regulation of cyclin D1 expression in breast, prostate and squamous cell carcinoma (51).
In the last part of our experimental approach, we determine at which step of the Wnt signaling cascade curcumin in order to understand the mechanisms by which curcumin leads to the decrease of Wnt transcriptional activity observed in 22rv1 cells. Co-transfection performed in the presence of mutated ß-catenin vector as well as Western blot analysis of ß-catenin expression (Fig. 7) revealed that curcumin has no effect on ß-catenin by itself, but acts on the transcriptionally active complex through the decrease of expression of Tcf-4 and of its co-activators CBP and p300 (53). This fits with previous published data reporting that curcumin is a potent in vitro inhibitor of p300/CBP (15,54) and that natural derivatives of curcumin (demethoxycurcumin and bisdemethoxycurcumin) are able to attenuate the expression of p300 co-activator and subsequently the Wnt transcriptional activity in human embryonic HEK293 cells (15).

In conclusion, we demonstrate that curcumin affects cell proliferation of androgen-dependent but not of androgen-independent prostate cancer cells through the modulation of the Wnt transcriptional complex and activity leading to a decrease of expression of ß-catenin targets genes highly implicated in cell cycle regulation. As the inhibition of the Wnt signaling appears at the level of downstream effectors, this avoids interference with the physiological role of the Wnt signaling pathway, so that curcumin appears as a considerable anti-neoplastic natural compound against androgen-dependent prostate cancer cells with reduced side-effects. Further investigations are needed to understand the inefficiency of curcumin on the Wnt signaling in androgen-independent prostate cancer cells and to evaluate the effect of promising curcumin analogs or derivatives on these cellular model representative of late stage of prostate cancer development.

Acknowledgements

M.H.T. is supported by Télévie grants (Fonds National de la Recherche Scientifique, Belgium). F.G. is a recipient of the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement 215009 RedCat. The research of M. Diederich at the Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (LBMCC) is financially supported by ‘Recherche Cancer et Sang’ foundation, by ‘Recherches Scientifiques Luxembourg’ asbl, by ’Een Häerz supported by ‘Recherche Cancer et Sang’ foundation, by Moléculaire et Cellulaire du Cancer (LBMCC) is financially supported by late stage of prostate cancer development.

References


