Dicoumarol down-regulates human *PTTG1/Securin* mRNA expression through inhibition of Hsp90

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Abstract

Securin, the natural inhibitor of sister chromatid untimely separation, is a protooncogene overexpressed in tumors. Its protein levels correlate with malignancy and metastatic proneness. Dicoumarol, a long-established oral anticoagulant, is a new Hsp90 inhibitor that represses *PTTG1/Securin* gene expression and provokes apoptosis through a complex trait involving both intrinsic and extrinsic pathways. Dicoumarol activity as an Hsp90 inhibitor is confirmed by smaller levels of Hsp90 clients in treated cells and inhibition of *in vivo* heat shock luciferase activity recovery assays. Likewise, established Hsp90 inhibitors (17-allylamino-geldanamycin and novobiocin) repress *PTTG1/Securin* gene expression. Also, overexpression of human Hsp90 in yeast makes them hypersensitive to dicoumarol. Both apoptosis and *PTTG1/Securin* gene repression exerted by dicoumarol in cancer cells are independent of three of the most important signaling pathways affected by Hsp90 inhibition: nuclear factor-κB, p53, or Akt/protein kinase B signaling pathways. However, effects on *PTTG1/Securin* could be partially ascribed to inhibition of the Ras/Raf/extracellular signal-regulated kinase pathway. Overall, we show that expression of *PTTG1/Securin* gene is Hsp90 dependent and that dicoumarol is a *bona fide* Hsp90 inhibitor. These findings are important to understand the mode of action of Hsp90 inhibitors, mechanisms of action of dicoumarol, and Securin overexpression in tumors. [Mol Cancer Ther 2008;7(3):474–82]

Introduction

Securin (*PTTG1/Securin* for its gene) is a cell cycle protein involved in the appropriate sister chromatid separation at mitotic anaphase. Its name derives from its ability to inhibit separase protease activity. Securin levels are elevated in rapidly proliferating cells and many tumor types (1). Furthermore, increased Securin amounts have been correlated to proliferation and metastasis by solid tumors (2). Recently, there had been some clues about the mechanisms regulating its gene expression (3–5), but the reasons behind its overexpression in malignant cells are still obscure. On the other hand, it has been shown that Securin interacts and modulates p53-transactivating functions *in vivo* and *in vitro* (6) and that p53 may be involved in Securin regulation under stress circumstances (7).

Dicoumarol is a coumarin derivative used medically in the past as an oral anticoagulant (8). As several other coumarin derivatives, it shows antiproliferative effects that are receiving increasing attention. Thus, its cytostatic and cytotoxic effects on melanoma cells (9) and pancreatic cancer cells (10) have been described. However, its mechanisms for apoptosis induction are unknown. Among its many effects, dicoumarol has been shown to be a competitive inhibitor of the flavoprotein NAD(P)H:quinone oxidoreductase-1 (*NQO1*, DT-diaphorase; ref. 11). However, the specificity of dicoumarol for *NQO1* is low. Other effects, such as inhibition of nuclear factor-κB (NF-κB) and c-Jun NH_2-terminal kinase (JNK) activation (12), have been reported.

Hsp90 is a chaperone with a large list of clients. These are mostly kinases involved in signal transduction and transcription factors. Inhibition of Hsp90 leads to destabilization of their protein clients. This translates into down-regulation of signaling pathways involved in proliferation, such as those integrated by Akt [phosphatidylinositol-3-kinase (PI3K)/Akt] and Raf-1 [Ras/Raf/extracellular signal-regulated kinase (ERK)], and repression of transcription driven directly by its clients or by downstream transcription factors, such as NF-κB (13, 14). Moreover, inhibition of chaperone activity induces cell death through activation of both intrinsic and extrinsic pathways of apoptosis (15). Hsp90 activity is increased in all cancers, and tumor cells seem to depend, to a greater extent, on Hsp90 activity than normal. This is believed to constitute the basis of the observed specificity of Hsp90 inhibitors towards neoplasias (16). Not surprisingly then, inhibitors of Hsp90 are among the most promising antitumoral new drugs.

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Several types of small molecules have been found to inhibit Hsp90. Geldanamycin and its derivatives are considered very specific and have reached clinical trial stage. Unfortunately, their limited solubility and potential hepatotoxicity may constitute problems when applied to patients (17). Novobiocin was the first coumarin found to inhibit Hsp90 (18). Since then, the search for other coumarin derivatives has rendered a few other compounds (19).

We describe here that both dicoumarol and conventional Hsp90 inhibitors repress expression of the *PTTG1/Securin* gene. Moreover, our work shows that dicoumarol inhibits *PTTG1/Securin* expression through a novel Hsp90 inhibitor activity. This activity contributes to explain dicoumarol ability to promote apoptosis, because inhibition of Hsp90 is a well-known elicitor of cell death. On the other hand, the ability to down-regulate Securin adds to the antineoplastic mechanisms of action accredited to Hsp90 inhibitors and suggests a mechanism that could help Securin overexpression in tumors.

Materials and Methods

Cell Lines, Reagents, and Treatments

Wild-type HCT116 human colon adenocarcinoma cells ($p53^{+/+}$ HCT116 and $p53^{-/-}$ HCT116) have been described previously (20). HCT116 cells were maintained in McCoy's 5A medium plus 10% fetal bovine serum. HeLa and Caco-2 cells were propagated in DMEM with 10% fetal bovine serum. Dicoumarol, SP600125, SB503960, and Z-VAD-fmk were from Sigma-Aldrich. For dicoumarol treatments, the drug was added to subconfluent cells for 24 h. Unless otherwise stated, Z-VAD-fmk and other inhibitors were added to cells 10 min before addition of dicoumarol.

DNA Constructs and Transfections

Plasmids carrying *IκBα (S32,36A)* and *RelA/p65* have been described elsewhere (21). Transfections were done using Jet-PEI (Qbiogene) according to the manufacturer's instructions. Total DNA was adjusted to 3 μg/well with pcDNA3.1. Under these conditions, between 40% and 60% of cells were routinely transfected. Twenty-four hours after transfection, cells were treated with dicoumarol or vehicle for a further 24-h period before protein extraction.

Luciferase Denaturation and Refolding Assays

A pcDNA3.1 firefly luciferase construct was transiently transfected into HCT116 cells. After 24 h, cells were incubated in the presence of inhibitors or vehicle for 30 min before the onset of the experiment. Heat shock denaturation of luciferase *in vivo* was done as described in ref. 22. Luciferase activity was measured using a luciferase assay kit (Promega) as per manufacturer's instructions.

Yeast Methods

Yeast (*Saccharomyces cerevisiae* strain W303-1a) was transformed with plasmids pG1, pHsp90β, or pHsp82-Flag (23) by the lithium acetate method (24). Transformants were grown to early stationary phase and diluted to ~4,000 cells/μL. Ten-fold serial dilutions were spotted onto Drop-Out defined medium agar plates supplemented with 20 μmol/L dicoumarol or vehicle. Plates were photographed after 3-day incubation at 30°C.

Western and Northern Blots

Cells were lysed and soluble proteins were harvested in RIPA buffer [25 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 10% glycerol, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS] plus protease inhibitors (Roche) and 1 mmol/L phenylmethylsulfonyl fluoride. Proteins were resolved on SDS-PAGE gels and transferred to nitrocellulose filters using standard procedures. Immunoblotting was done using the following antibodies: anti-Bcl-2 (Alexis), anti–poly(ADP-ribose) polymerase-1 (PARP-1; Roche), anti-β-actin and tubulin (Sigma-Aldrich), anti-phospho-Ser^{15} p53, activated caspase-3, and phospho-Thr^{180}/Tyr^{182} p38 (Cell Signaling Technologies), anti-caspase-3 (*CPP32*) and caspase-7 (BD PharMingen), anti-p53 (DO-1), Mcl-1 (S-19), Bcl-x_L (H-62), Bid (FL-195), phospho-Tyr^{204}-ERK (E-4), and phospho-Thr^{183}/Tyr^{185}-JNK (G-7; Santa Cruz Biotechnologies), anti-IκB, and RelA/p65 antibodies have been described previously (21). Immunoblots were developed using a horseradish peroxidase–conjugated secondary antibody and chemiluminescence detection (ECL kit; Amersham Biosciences).

Northern blots were done using 15 μg total RNA separated on formamide-agarose gels and transferred to nylon membranes. Blots were hybridized with a full-length *PTTG1* probe (25). Bands were detected on X-ray films.

When appropriate, films were scanned on a Bio-Rad GS-800 densitometer. Protein half-life was calculated from band intensity applying the equation: $t_{1/2} = \ln 2 / K$, where K is the exponent of the exponential decay function: $C = C_0 e^{-kt}$.

Flow Cytometry

Floating and adherent cells were stained with propidium iodide and processed for flow cytometry analysis on a Coulter Epics XL apparatus as described (26). The number of apoptotic cells was determined as the percentage of the population showing sub-G_0-G_1 DNA content. Proportion of cells in G_1, S, and G_2-M phases was determined using the algorithm described by Watson et al. (27).

Semiquantitative Reverse Transcription-PCR

Total RNA (1 μg) was subjected to cDNA synthesis using a $polyT_{(18)}$ primer and SuperScript RNase H Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Final cDNA (1 μL) was subjected to PCR using primers GAACTCGAGAAATTTAAATATCTATGTC (reverse) and CTAGAATTCGAATGGCTACTCTGATC (forward). PCR was run for a total of 20 cycles. Primers annealing on the glyceraldehyde-3-phosphate dehydrogenase cDNA (reverse GAAGGGGTCATTGATGGCAA and forward TGGGGAAGGTGAAGGTCGGA) were used to check equal cDNA loading onto PCR mixes. Saturation of the reaction and specificity of primers were checked using a 3-fold excess of cDNA obtained from $Sec^{-/-}$ HCT116 cells.

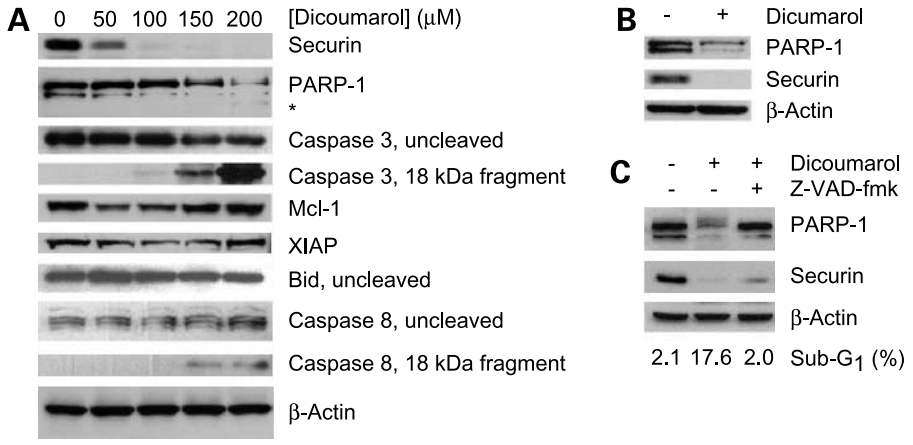

Figure 1. Dicoumarol induces apoptosis and Securin down-regulation in a dose- and time-dependent manner. **A,** dose-response effect of dicoumarol on HCT116 cells. Cells were incubated in the presence of the indicated concentrations of dicoumarol for 24 h and then analyzed by Western blot for changes in the amount of Securin, PARP-1, and other regulators of apoptosis in total cell lysates. **B,** apoptosis and Securin levels in cells lacking NQO1 activity. Caco-2 cells were incubated with 200 μmol/L dicoumarol for 24 h and their whole-cell homogenates were analyzed for changes in PARP-1 and Securin by Western blot. **C,** death induction is caspase dependent. Cells were incubated for 24 h in the presence or absence of 150 μmol/L dicoumarol and 50 μmol/L Z-VAD-fmk. Whole-cell lysates were analyzed for PARP-1 and Securin by Western blot. *Asterisk*, the 84-kDa band corresponding to cleaved PARP-1. As a loading control, β-actin levels are shown.

Other Methods

Protein contents were quantified using a Bio-Rad Protein Assay kit (Bio-Rad) according to the manufacturer's instructions and using bovine serum albumin as a standard. Experiments were typically done in triplicate. Unless otherwise stated, a representative experiment is shown in each case.

Results

Dicoumarol Reduced Securin Protein Levels and Induced Apoptosis

While working on the influence of Securin on p53 stability, we observed that treatment of cells with dicoumarol reduced Securin protein levels. Detailed studies showed that this effect was dose and time dependent in HCT116 cells and concomitant to apoptosis induction as assessed by marker analysis (Fig. 1A; data not shown). In our hands, the early apoptosis marker PARP-1 appeared as a double band (of ~110 and 120 kDa, respectively) maybe due to posttranslational modifications (28). The low affinity of our antibody for the 84-kDa form of PARP-1 prevented us from following the apoptotic process by the appearance of this form, but we could relate it to the disappearance of the 110- to 120-kDa unproteolyzed doublet. As a complement, we routinely analyzed the appearance of cleaved (active) forms of caspase-3. Flow cytometry analysis also showed a dose-dependent increase in the proportion of cells bearing sub-G_1 DNA contents (Table 1).

Characteristics of apoptosis were looked into in detail (Fig. 1A). In particular, we attempted to determine if the apoptotic pathway was intrinsic or extrinsic. Active caspase-3 (common to both pathways) was detected at concentrations of dicoumarol above 100 μmol/L and its levels rose with increasing concentrations of the drug. Reduced levels of Mcl-1, a regulator of the intrinsic branch of apoptosis, were observed at concentrations as low as 50 μmol/L but were similar to control at concentrations above 150 μmol/L. Similarly, X-linked inhibitor of apoptosis, another regulator of the same pathway branch of apoptosis, showed minimal levels at 100 μmol/L dicoumarol but recovered at greater concentrations. No changes were observed for caspase-7, Bcl-2, or Bcl-x_L (data not shown). Proteins involved in the death receptor pathway of apoptosis appeared in their active forms at greater concentrations. Thus, the active p18 form of caspase-8 was found at concentrations above 150 μmol/L; at this same concentration, amounts of Bid were observed to decrease. On the whole, markers of activation of the intrinsic pathway of apoptosis were evident at low concentrations of dicoumarol, whereas they were substituted by markers of the extrinsic pathway at high concentrations.

Table 1. Effect of dicoumarol on death induction and cell cycle in HCT116 cells [average ± SE (*n* = 3)]

Dicoumarol (μmol/L)	Sub-G_1	G_1	S	G_2-M
Vehicle	1.4 ± 0.1	31.4 ± 0.6	51.8 ± 1.3	16.8 ± 0.9
50	2.6 ± 0.4	45.8 ± 1.6	43.5 ± 1.7	10.7 ± 0.4
100	7.9 ± 0.8	57.2 ± 0.9	31.3 ± 1.1	11.5 ± 1.8
150	18.8 ± 1.6	44.9 ± 0.9	41.8 ± 2.2	13.3 ± 2.3
200	30.7 ± 0.5	37.9 ± 0.2	49.0 ± 1.3	13.1 ± 1.2

NOTE: HCT116 cells were incubated with the indicated concentrations of dicoumarol for 24 h in McCoy's medium. Cell cycle profiles were obtained by propidium iodide staining and flow cytometry analysis. Cell cycle data are percentages of cells in each phase with respect to total cycling cells. Sub-G_1 data are percentages of cells with DNA contents lower than G_1 with respect to total cells.

Concentrations of dicoumarol above 100 μmol/L were effective in reducing Securin contents to undetectable levels in 24 h in HCT116 and similar results were obtained in HeLa cells (data not shown). Dicoumarol inhibits NQO1, but Caco-2 cells did also prove sensitive to dicoumarol, although this cell line lacks any NQO1 activity (Fig. 1B; ref. 29). Despite several attempts, we could not detect p18 fragments of caspase-3 in this last cell line (data not shown).

Securin is a cell cycle–regulated protein with minimal levels in G_1 (30); hence, we tested if dicoumarol provoked cell cycle arrest in G_1 phase in HCT116. Increasing amounts of dicoumarol translated into a slightly greater proportion of cells in G_1 (Table 1). However, increases in G_1 did not justify differences in Securin protein levels (Fig. 1A; Table 1).

Apoptosis by dicoumarol could be prevented by cotreatment with a general inhibitor of caspases, such as Z-VAD-fmk, as seen either as PARP-1 degradation or as proportion of cells in sub-G_1 (Fig. 1C). However, Securin levels were not recovered by inhibition of caspases, although a tiny increase was noticed.

Repression of PTTG1/Securin Expression

Reduced Securin protein levels could be caused by gene repression or increased proteolysis. Hence, we quantitated both mRNA levels and protein half-life for Securin. Northern blots showed that mRNA amounts from PTTG1/Securin gene were reduced in a dose-dependent manner in dicoumarol-treated HCT116 cells (Fig. 2A). Reductions in PTTG1/Securin mRNA correlated with those observed for protein levels (Fig. 1A). In HeLa cells, amounts of PTTG1/Securin mRNA equally proved to be sensitive to dicoumarol. On the other hand, HCT116 cells treated with a low dose of dicoumarol for 24 h (50 μmol/L) showed no significant differences in protein turnover rate (39.2 min for dicoumarol-treated cells versus 34.1 min for control cells; Fig. 2B). Similar results were obtained using 400 μmol/L dicoumarol for 4 h (data not shown).

Lack of Involvement of NF-κB, Reactive Oxygen Species, and p53

Dicoumarol has been shown to be a potent inhibitor of the NF-κB pathway. Indeed, HCT116 cells transfected with a HIV-derived NF-κB-responsive promoter fused to luciferase showed decreased expression in dicoumarol-treated cells (data not shown). The interest of this pathway is highlighted by the presence of a putative binding site for this transcription factor on the *PTTG1/Securin* promoter. Transfection of *RelA/p65* did not ameliorate dicoumarol-induced repression of *PTTG1/Securin* or apoptosis, despite its clear influence on IκBα levels, an NF-κB-responsive gene itself (31). Similarly, overexpression of a nonphosphorylatable IκBα mutant did not mimic dicoumarol effects on Securin down-regulation or death induction (Fig. 3A).

It has been reported that dicoumarol induces cell death through reactive oxygen species generation in pancreatic cells but that it could be prevented by addition of antioxidants (10). In our hands, addition of ascorbate, a well-known superoxide scavenger, or *N*-acetylcysteine did not ameliorate Securin down-regulation or apoptosis induction by 150 μmol/L dicoumarol in the colorectal cell line HCT116 (Fig. 3B). *N*-acetylcysteine produced a slight decrease in caspase-3 p18 levels, but this was not accompanied by comparable alterations in sub-G_1 cells, Securin or PARP-1 levels. Similar results were obtained at a lower dose (100 μmol/L dicoumarol; data not shown). On the other hand, antioxidants were used in a wide range of concentrations; Fig. 3 shows data using the highest concentration of antioxidants not showing toxic effects.

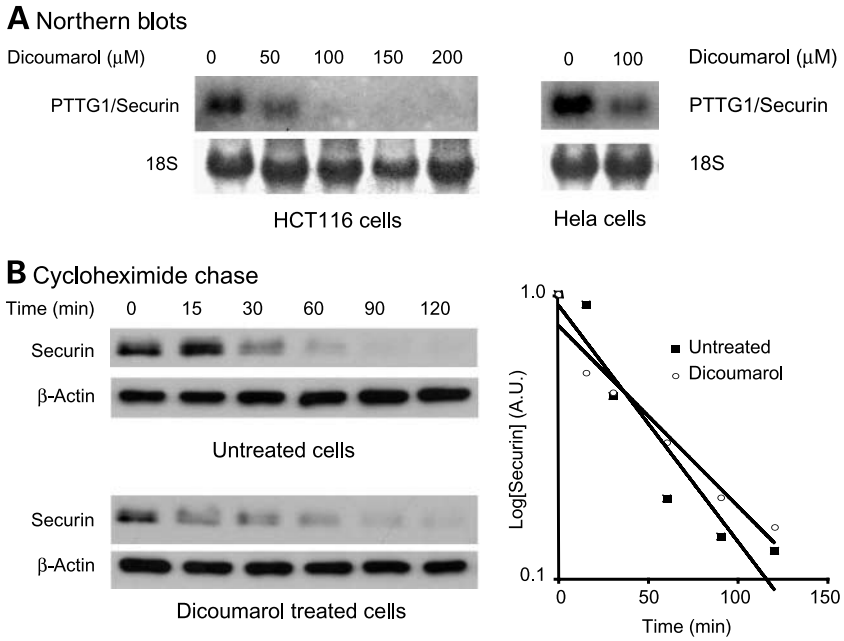

Figure 2. Dicoumarol represses PTTG1/Securin mRNA expression. **A,** analysis of mRNA abundance. Total RNA was extracted from HCT116 (*left*) or HeLa cells (*right*) treated with the indicated concentrations of dicoumarol for 24 h. Levels of PTTG1/Securin mRNA were analyzed by Northern blot. Methylene blue staining corresponding to the 18S rRNA is shown as a loading control. **B,** determination of Securin protein half-life. Western blots (*left*) and semilogarithmic plot of these data (*right*). HCT116 cells were treated with or without 50 μmol/L dicoumarol for 24 h. Thereafter, cells were added cycloheximide (40 μg/mL) to stop protein translation. Total cell extracts were obtained from cells collected at the indicated times. Calculated half-lives based on band intensities were 34.1 min for untreated controls and 39.2 min for dicoumarol-treated cells.

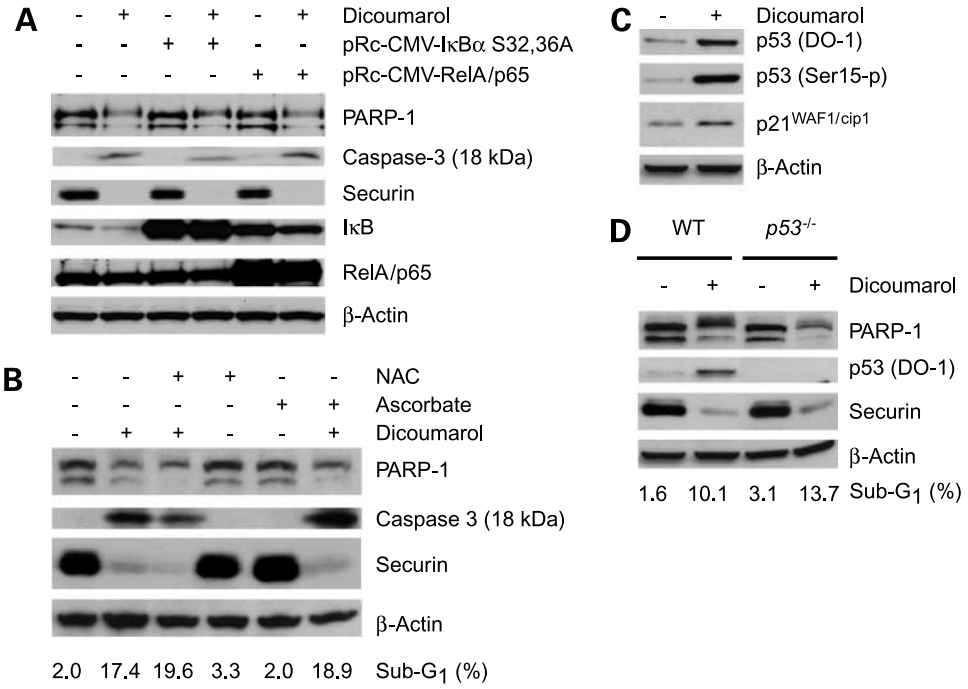

Figure 3. Analysis of pathways associated to dicoumarol. **A,** NF-κB is not involved in apoptosis or Securin down-regulation. HCT116 cells were transfected with 1 μg of the indicated plasmids and up to 3 μg with pcDNA3.1. After 24 h, cells were incubated in the presence or absence of 100 μmol/L dicoumarol for a further 24-h period. Subsequently, cell proteins were extracted and analyzed by Western blot using the indicated antibodies. **B,** *N*-acetylcysteine or ascorbate are ineffective to reduce cell death. HCT116 cells were treated with dicoumarol (150 μmol/L) or vehicle in the presence or absence of *N*-acetylcysteine (1 mmol/L) or sodium ascorbate (200 μmol/L) for 24 h. Cells were then collected and analyzed by Western blot or flow cytometry. Amounts of sub-G_1 cells are given as a percentage from total cells analyzed by propidium iodide staining and flow cytometry. **C,** presence of active p53 in dicoumarol-treated HCT116 cells. Total cell protein extracts from dicoumarol-treated (100 μmol/L, 24 h) or vehicle-treated cells were analyzed by Western blot using the indicated antibodies. **D,** active tumor suppressor p53 does not cause apoptosis or Securin down-regulation. HCT116 or *p53*$^{-/-}$ HCT116 cells were treated with 100 μmol/L dicoumarol or vehicle for 24 h. Cell extracts were analyzed by Western blot using PARP-1, DO-1, Securin, or anti-β-actin antibodies.

The stress-responsive p53 transcription factor has been shown to influence *PTTG1/Securin* gene expression on DNA damage (7) and dicoumarol is known to exacerbate DNA-damaging conditions (32). Therefore, we tested if p53 had any influence on apoptosis and Securin levels. Indeed, p53 was found activated and stabilized in dicoumarol-treated cells (Fig. 3C). However, this activation was minor when compared with that attainable by DNA damage with doxorubicin (data not shown). Thus, $p21^{CIP1}$ levels were only modestly increased in dicoumarol-treated cells. Experiments using a *p53*$^{-/-}$ HCT116 cell line showed that dicoumarol had no differential effects on apoptosis or Securin protein levels when compared with its wild-type parental (Fig. 3D). This agrees with what is observed in *p53-null* Caco-2 and HeLa cells (Figs. 1B and 2A, respectively).

Hsp90 Inhibitors and *PTTG1/Securin* Expression

Some coumarins have been shown to act as Hsp90 inhibitors (18). To test if dicoumarol could inhibit this chaperone, we assessed if dicoumarol mimicked known inhibitors of Hsp90. Indeed, dicoumarol reduced the levels of two well-known markers of Hsp90 inhibition: Akt and ErbB-2/HER-2 (Fig. 4A). This reduction was also observed when Akt was expressed exogenously from a constitutive promoter, ruling out any effects of dicoumarol on Akt gene expression (Fig. 5A). Similarly, treatment of HCT116 cells with 17-allylamino-geldanamycin (17AAG) or novobiocin translated into reduced levels of Securin polypeptides on Western blots (Fig. 4A). Likewise, dicoumarol and both Hsp90 inhibitors repressed mRNA expression from *PTTG1/Securin* gene, compared with untreated controls, as seen on semiquantitative reverse transcription-PCR assays (Fig. 4B). Overall, effects of dicoumarol, 17AAG, and novobiocin were somewhat variable when compared with each other. However, differences in inhibitory effect profiles are common among Hsp90 inhibitors and may reflect differences in mechanisms of action on the Hsp90 molecule (33).

To further confirm dicoumarol as an Hsp90 inhibitor, we transformed yeast cells with expression plasmids carrying human Hsp90β isoform or its most closely related gene in yeast, Hsp82. Drop tests showed that overexpression of the human isoform conferred hypersensitivity towards dicoumarol in the growth medium compared with empty vector-transformed cells. Similarly, but to a lesser extent, did overexpression of its yeast orthologue, Hsp82 (Fig. 4C).

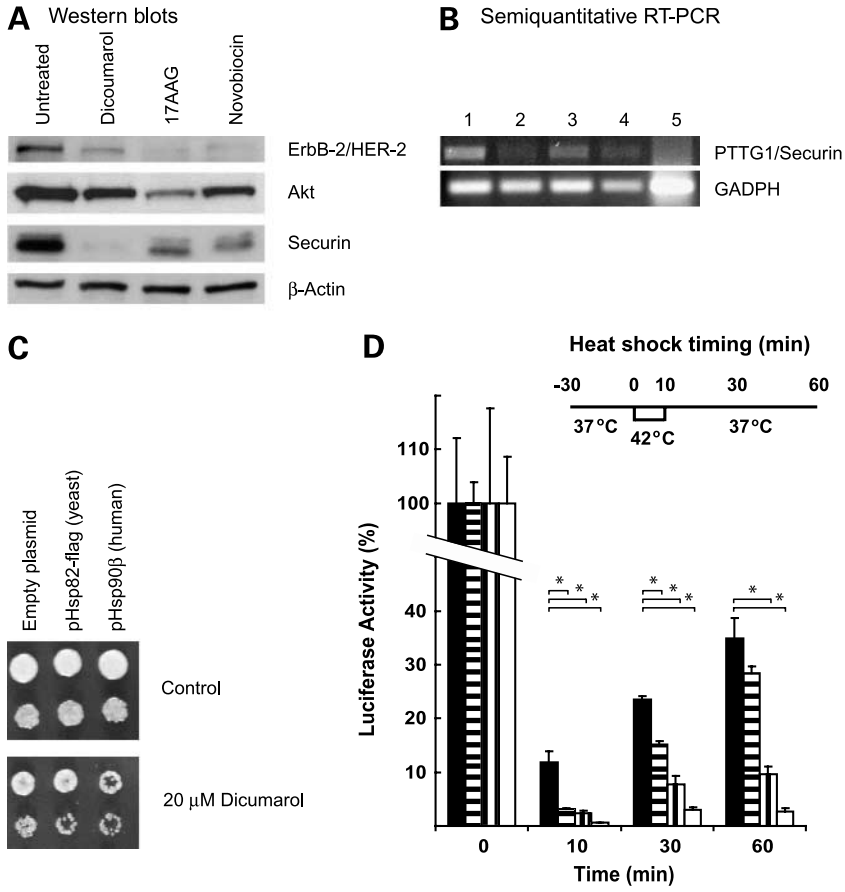

Figure 4. Dicoumarol inhibits Hsp90. **A,** dicoumarol brings down levels of two Hsp90 activity markers. HCT116 cells were treated with vehicle, dicoumarol (150 μmol/L), 17AAG (1 μmol/L), or novobiocin (0.8 mmol/L) for 24 h. After this period, total cell proteins were extracted and analyzed by Western blot. ErbB-2/HER-2 and Akt were used as markers of Hsp90 inhibition. **B,** Hsp90 inhibitors repress PTTG1/Securin mRNA expression. Cells were treated as in **A** and their RNA was extracted. cDNA (1 μL) was subjected to semiquantitative PCR (see Materials and Methods). *Lane 1*, control; *lane 2*, 150 μmol/L dicoumarol; *lane 3*, 1 μmol/L 17AAG; *lane 4*, 0.8 mmol/L novobiocin; *lane 5*, *Sec*$^{-/-}$ HCT116 cells (3 μL cDNA). **C,** human Hsp90β makes yeast cells hypersensitive to dicoumarol. *S. cerevisiae* cells (strain W303-1a) were transformed with the indicated expression plasmids. Cultures were grown to early stationary phase and 2.5 μL from a dilution series were spotted onto control or 20 μmol/L dicoumarol-supplemented SD medium. Plates were photographed after 3 d growth at 30°C. **D,** dicoumarol effect on luciferase activity loss and recovery. HCT116 cells were transfected with 1 μg of a pcDNA3.1-luciferase construct 24 h before the experiment. Cells were pretreated with Hsp90 inhibitors for 30 min (250 μmol/L dicoumarol, 2.5 μmol/L 17AAG, or 1.5 mmol/L novobiocin) and luciferase was heat denatured by transfer of cultures to 42°C for 10 min. Cells were then left to recover at 37°C for the indicated times. Firefly luciferase activity was assayed on whole-cell lysates. *, $P \leq 0.05$ (unpaired *t* tests). *Solid columns*, vehicle; *horizontal stripes*, dicoumarol; *vertical stripes*, 17AAG; *open columns*, novobiocin.

This last protein was also seen to bind dicoumarol (Supplementary Material).[3]

Firefly luciferase is sensitive to heat shock and denatures when cells are transiently incubated at 42°C. The extent of its denaturation and its recovery through refolding has been shown to be dependent on Hsp90; this feature has been used to identify small-molecule inhibitors (22). HCT116 cells transiently expressing firefly luciferase were treated with dicoumarol (250 μmol/L), 17AAG (2.5 μmol/L), or novobiocin (1.5 mmol/L). After shifting cells to denaturating temperature, luciferase activity dropped to ~12% of the initial value in control cells. This reduction was significantly greater in all inhibitor-treated cells (Fig. 4D), activity levels ranging between 3.1% and 0.6%. Twenty and 50 minutes after the end of the heat shock, drugs were still found to affect recovery of luciferase activity, albeit dicoumarol was the least effective (Fig. 4D).

Effect of Dicoumarol on Akt and Mitogen-Activated Protein Kinases

Protein kinases are the most common clients of Hsp90 and regulate many functions related to cancer progression. In breast cancer cells, insulin and insulin-like growth factor-I have been reported to regulate Securin protein levels through the phosphatidylinositol-3-kinase/Akt pathway (34). On its turn, Akt is a typical Hsp90 client, and as shown in Fig. 4A, its levels decline in the presence of dicoumarol in the growth medium. This effect was also observed in HeLa and Caco-2 cells (data not shown). We tested the involvement of this pathway on dicoumarol-induced down-regulation of Securin. We transfected HeLa cells with a constitutively active or a dominant-negative form of Akt (myristoylatable and K179M mutants, respectively). Protein levels of Akt in transfected HeLa cells treated with dicoumarol were remarkably smaller than those of their untreated controls (Fig. 5A). This is in agreement with destabilization of these proteins through Hsp90 inhibition. Despite this, levels of Akt(myr) in the presence of dicoumarol were ~15-fold greater than those observed in control cells. However, this did not alter dicoumarol-induced repression of Securin. In line with this, overexpression of a dominant-negative form of Akt on its own did not have any effects on the Securin protein levels. Similar results were obtained using HCT116 cells (data not shown). Our cell lines express *PTTG1/Securin* independently of the insulin-like growth factor-I/ phosphatidylinositol-3-kinase/Akt pathway (Supplemental data).[3]

The Ras/Raf/MEK/ERK pathway is one of the most important pathways in tumor cell proliferation, and at least

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

one its components, Raf-1, is destabilized if Hsp90 is inhibited (13). Although not as common, effects of Hsp90 inhibition on other mitogen-activated protein kinases (MAPK), that is, p38MAPK and JNK, have also been reported (35, 36). On the other hand, inhibition of JNK activation has already been described as one of the effects of dicoumarol (12). HCT116 cells treated with dicoumarol showed decreased levels of active ERK1/2 and JNK but negligible differences in p38MAPK compared with untreated cells (Fig. 5B). When HCT116 cells were treated with PD98059, a specific inhibitor of MEK1/2, Securin levels were moderately affected (Fig. 5C). Also, PD98059 seemed to enhance apoptosis by dicoumarol as seen on PARP-1 and caspase-3 p18 levels. However, addition of PD98059 alone did not provoke the appearance of the active fragment of caspase-3 or degradation of PARP-1 (Fig. 5C). On their turn, no differential effects on Securin were discernible when cells were treated with inhibitors SB203580 (p38MAPK) or SP600125 (JNK) alone. When in combination with dicoumarol, only a minor increase in caspase-3 p18 levels were observed with SB203580 (Fig. 5D).

Discussion

Securin is seen as an increasingly important protein in cancer. The regulation of its expression may be of great importance to understand and control metastatic processes. However, lack of mutations in its promoter (37) points to deregulation of cell signaling as the key to understand Securin overexpression in tumors. On its turn, dicoumarol is seen progressively more as a putative useful drug in cancer due to its apoptosis promoting ability.

We show here that *PTTG1/Securin* mRNA and protein levels can be modulated by drugs inhibiting Hsp90, such as 17AAG or novobiocin, and that dicoumarol is a previously unrecognized inhibitor of this chaperone. The former claim is supported by experiments showing Securin down-regulation by Hsp90 targeting drugs. Reduction in the levels of Hsp90 clients such as Akt or ErbB-2/HER-2, yeast hypersensitivity to dicoumarol when human Hsp90β is overexpressed, inhibition of Hsp90-dependent recovery of luciferase activity after heat shock, and physical interaction between dicoumarol and yeast Hsp82 sustain the latter. Also, apoptosis induction by dicoumarol appears related to Hsp90 inhibition. The mechanism linking Hsp90 inhibition and *PTTG1/Securin* repression can be ascribed partially to inhibition of the Ras/Raf/ERK pathway. Apart from this, we can also conclude that some of the most likely players, NF-κB, p53, or phosphatidylinositol-3-kinase/Akt pathways, are in fact not involved in the transcriptional regulation of Securin by dicoumarol.

Insulin-like growth factor receptor signaling has been pointed out as one of the regulators of Securin expression both in breast tumor cells and in astrocytes (34, 38). Akt was pointed as the major player in relation to *PTTG1/Securin* gene regulation in both reports, whereas Ras/Raf/ERK had a lesser influence. Both pathways are sensitive to

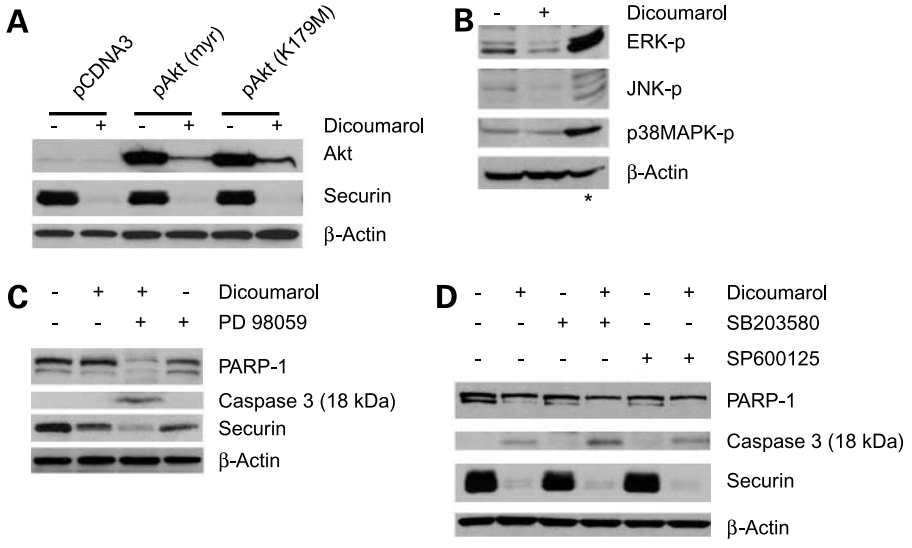

Figure 5. Ras/Raf/ERK pathway, but not Akt, affects PTTG1/Securin expression and dicoumarol-induced apoptosis. **A,** changes in Akt do not alter Securin expression. HeLa cells were transfected with 1 μg of the indicated plasmids and up to 3 μg of pcDNA3.1. After 24 h, cells were incubated in the presence or absence of 100 μmol/L dicoumarol for a further 24-h period before total cell protein extraction and analysis by Western blot using the indicated antibodies. **B,** dicoumarol affects some MAPKs. Extracts from cells treated or untreated with 100 μmol/L dicoumarol for 24 h were analyzed by Western blot for levels of active MAPKs and β-actin. *Asterisk*, cells treated with 0.5 mol/L sorbitol for 30 min (positive control). **C,** inhibition of the Ras/Raf/ERK pathway influences Securin levels. HCT116 cells were treated with MEK1/2 inhibitor PD98059 (50 μmol/L) for 24 h in the presence or absence of 100 μmol/L dicoumarol. Total cell extracts were analyzed by Western blot using the indicated antibodies. **D,** inhibition of JNK or p38MAPK does not alter dicoumarol-induced apoptosis or Securin levels. Cells (HCT116) were treated with or without dicoumarol for 24 h in the presence or absence of JNK (SP600125, 10 μmol/L) or p38MAPK (SB203580, 30 μmol/L) specific inhibitors. Total cell extracts were analyzed by Western blot using the indicated specific antibodies.

Hsp90 inhibition because Raf-1, PDK1, and Akt itself are Hsp90 clients (13). However, *PTTG1/Securin* expression is insensitive to both insulin-like growth factor-I and Akt in HCT116 (Supplementary Material;[3] data not shown). On the other hand, Ras/Raf/ERK pathway is indeed affected by dicoumarol, and in concordance with the above-mentioned studies, it has a partial influence on Securin levels. All in all, the expression of *PTTG1/Securin* may be determined chiefly by other signaling events in HCT116 and HeLa cells and opens the field to further studies.

Dicoumarol is considered an inhibitor of NF-κB (12) and this transcription factor has been shown imperative for the growth and survival of some tumor cell lines (39, 40). On the other hand, it has recently been proposed that dicoumarol enhances reactive oxygen species production and promotes apoptosis (10). In agreement with these data, intracellular levels of superoxide and hydrogen peroxide increased with dicoumarol treatment (data not shown). However, in our experimental settings, apoptosis and *PTTG1/Securin* repression were insensitive to both antioxidants and alterations in NF-κB signaling (Figs. 4B and 5A). Paradoxically, Securin levels have been revealed sensitive to hydrogen peroxide stress in a recent report (41). However, hydrogen peroxide–induced Securin reduction needs concentrations of oxidant similar to those provoking Hsp90 degradation (42). This may account for the decay of Securin protein levels in the study by D'Angiolella et al.

The mechanism of apoptosis, either extrinsic or intrinsic, is important in pharmacology and in the clinical practice because it may critically affect the time needed for the execution of the death program and its extensibility to surrounding cells (43). In our hands, apoptosis followed a complex trait: whereas at low concentrations of dicoumarol the intrinsic/mitochondrial pathway seemed to be the main mechanism, this switched to extrinsic as dicoumarol concentration increased. Inhibition of Hsp90 has been shown to promote apoptosis through both intrinsic and extrinsic pathways. Inhibition of Hsp90, among other effects, destabilizes RIP, provoking resistance to tumor necrosis factor–mediated apoptosis, and facilitates cytochrome *c*/dATP–mediated oligomerization of Apaf-1 (44). Dual activation of both intrinsic and extrinsic pathways of apoptosis fits broadly with Hsp90 inhibition being a major player in dicoumarol-induced apoptosis. However, having in mind that dicoumarol has numerous off-target effects, it is very likely that other mechanisms contribute to its apoptogenic ability.

Hsp90 activity may be one of the players in promoting elevated levels of Securin in cancer cells. This may be accomplished through stabilization of transcription factors or signaling molecules. Further work should be done to pinpoint the Hsp90-sensitive step that governs PTTG1/Securin transcription. In parallel, Hsp90 inhibitors are known to produce aneuploidy through inhibition of Polo-like kinase (45). Being Securin one of the regulators of proper sister chromatid separation, a future line of research could be to determine if inhibition of Hsp90 adds to the aneuploidy-promoting action of these drugs through down-regulation of Securin protein levels in mitotically active cells.

Dicoumarol is a small, symmetric molecule produced conceptually by the condensation of two 4-hydroxy-5-methylcoumarin residues. Despite some coumarins, that is, novobiocin, coumermycin A1, and some of their derivatives, are known to inhibit Hsp90 (18), to date there is no evidence that coumarins, as a compound family, are Hsp90 inhibitors. In this respect, this report presents dicoumarol as the simplest coumarin known to date to inhibit Hsp90 and may be a starting point for screening other coumarin derivatives. Interestingly, several naturally produced coumarins have antiproliferative and anti–drug resistance effects (46). This latter effect may be connected to P-glycoprotein, the multidrug efflux pump, being an Hsp90 client (47). On the other hand, novobiocin and other coumarins have been shown to inhibit Hsp90 activity through an ATP-binding site located at the COOH terminus of the protein (48). It would be interesting to study if dicoumarol targets this same domain. At any rate, the present data show that repression of Securin gene expression is a common feature of Hsp90 inhibitors irrespective of its mode of action.

Finally, inhibition of Hsp90 by dicoumarol gives a different perspective to understand the effects of this compound in cells.

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