

Genistein Inhibits Matrix Metalloproteinase Type 2 Activation and Prostate Cancer Cell Invasion by Blocking the Transforming Growth Factor β -Mediated Activation of Mitogen-Activated Protein Kinase-Activated Protein Kinase 2–27-kDa Heat Shock Protein Pathway

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ABSTRACT

Genistein is a candidate cancer chemopreventive drug being tested in clinical trials. We have shown that genistein blocks prostate cancer (PCa) cell invasion, that p38 mitogen-activated protein (MAP) kinase regulates activation of matrix metalloproteinase type 2 (MMP-2) and cell invasion, and that genistein prevents transforming growth factor β (TGF β) from activating p38 MAP kinase. More recently, we identified MAP kinase-activated protein kinase 2 (MAPKAPK2) and the 27-kDa heat shock protein (HSP27) as downstream regulators of p38 MAP kinase. However, MAPKAPK2 and HSP27 can be regulated by factors other than p38 MAP kinase, and HSP27 is up-regulated during PCa progression. The current study was undertaken to examine the role of MAPKAPK2 and HSP27 in modulating genistein-mediated regulation of PCa cell invasion. Genistein inhibited TGF β -mediated phosphorylation of MAPKAPK2 and HSP27. Inhibitory effects by genistein upon cell signaling, inhi-

bition of MMP-2, and inhibition of invasion were retained when both PC3 and PC3-M cells were transfected with either wild-type MAPKAPK2 or HSP27. However, transfection with dominant-negative MAPKAPK2 or nonphosphorylatable mutant HSP27 led to decreases in cell invasion and to abrogation of responsiveness to either TGF β -mediated increases or genistein-mediated decreases in MMP-2 and cell invasion. It is noteworthy that, after transfection with constitutive active MAPKAPK2 or with pseudophosphorylated HSP27, levels of MMP-2 activation and cell invasion were high and overcame any inhibitory effect of genistein. These findings demonstrate that genistein-mediated inhibition of cell invasion rests upon blocking activation of the MAPKAPK2-HSP27 pathway, and that its activation during cancer progression has the potential to mitigate therapeutic efficacy.

Among American men, prostate cancer (PCa) is the most common invasive malignancy (Jemal et al., 2005). Despite increased surveillance and early detection, PCa is still ranked second as a cause of cancer-related death. New forms of therapy are thus needed. Genistein (4',5,7-trihydroxyflavone) is a predominant constituent of soy and is a potential PCa chemopre-

ventive agent (Davis et al., 2000; Bergan et al., 2001). Phase 1 trials of genistein in men with PCa have been completed by us (Takimoto et al., 2003) and others (Mills et al., 1989; Knight and Eden, 1996; Busby et al., 2002; Dalais et al., 2004; Fischer et al., 2004), and phase 2 efficacy trials are under way. To optimize clinical development, it is important to understand the molecular pharmacology of genistein, particularly as it relates to efficacy in PCa.

Genistein inhibits PCa cell detachment and cell invasion in preclinical mechanistic studies (Bergan et al., 1996; Liu et al., 2000; Sarkar and Li, 2002; Huang et al., 2005). Because cell detachment and invasion represent initial steps in the

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ABBREVIATIONS: PCa, prostate cancer; genistein, 4',5,7-trihydroxyflavone; TGF β , transforming growth factor β ; MAP, mitogen-activated protein; MMP-2, matrix metalloproteinase type 2; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; HSP27, 27-kDa heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild-type; MK2 WT, wild-type pcDNA3mycMK2; MK2 DN, dominant-negative kinase inactive mutant of pcDNA3mycMK2; MK2 EE, constitutive active mutant of pcDNA3mycMK2; HSP27 3D, pseudophosphorylated mutant of pcDNA3.1-HSP27; HSP27 3G, nonphosphorylatable mutant of pcDNA3.1-HSP27; TBST, Tris-buffered saline/Tween 20; BSA, bovine serum albumin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; VC, vector control.

metastatic cascade (Eccles, 2005; Thompson et al., 2005), genistein seems to have antimetastatic potential in PCa. In particular, through a series of studies, we demonstrated that genistein inhibits PCa cell detachment by initiating complex formation between focal adhesion kinase and $\beta 1$ -integrin, resulting in increased formation of focal adhesion complexes (Bergan et al., 1996; Kyle et al., 1997; Liu et al., 2000). In related studies, we showed that the TGF β signaling pathway was a key regulator of PCa cell adhesion and invasion (Jovanovic et al., 2001; Liu et al., 2002), and went on to show that p38 MAP kinase was necessary for TGF β -mediated regulation of PCa cell adhesion (Hayes et al., 2003). After demonstrating that TGF β -mediated activation of matrix metalloproteinase type 2 (MMP-2) and cell invasion were regulated by p38 MAP kinase, we demonstrated that genistein blocked TGF β -mediated activation of p38 MAP kinase, MMP-2, and cell invasion (Huang et al., 2005).

We have most recently demonstrated that p38 MAP kinase activates mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), which in turn activates the 27-kDa heat shock protein (HSP27), and that both MAPKAPK2 and HSP27 regulate MMP-2 activation and cell invasion (Xu et al., 2006). Because both MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase, we hypothesized that they were involved in genistein-mediated modulation of PCa cell motility. However, this was not clear, because MAPKAPK2 and HSP27 can be activated by proteins other than p38 MAP kinase (Landry et al., 1992; Ludwig et al., 1996; Maizels et al., 1998; Butt et al., 2001; Yannoni et al., 2004; Doppler et al., 2005). Furthermore, p38 MAP kinase can activate proteins other than MAPKAPK2 and HSP27 (Shi and Gaestel, 2002; Platanius, 2003). It is also important that HSP27 is up-regulated during PCa cell progression (Cornford et al., 2000) and facilitates androgen-independent progression after androgen ablation (Rocchi et al., 2005). Such a situation may mitigate the therapeutic effects of genistein. The current study was therefore undertaken to elucidate the role of MAPKAPK2 and HSP27 in mediating and modulating the effects of genistein upon the regulation of PCa cell invasion.

Materials and Methods

Materials. Phosphatase inhibitor cocktails I and II, Genistein (4',5,7-trihydroxyisoflavone), and gelatin (used in invasion assays) were purchased from Sigma (St Louis, MO). Genistein was stored as stocks in dimethyl sulfoxide and was thawed just before use. Antibodies were obtained from the following sources: p38 MAP kinase (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-p38 MAP kinase (recognizes Thr180 and Tyr 182), phospho-MAPKAPK2 (recognizes Thr334), phospho-HSP27 (recognizes Ser82), MAPKAPK2, and HSP27 (Cell Signaling Technology, Danvers, MA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay Designs, Ann Arbor, MI); anti-mouse Ig-horseradish peroxidase and anti-rabbit Ig-horseradish peroxidase, were part of the ECL Western Blotting System (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and were used to detect proteins in Western blots. Recombinant human TGF $\beta 1$ (R&D Systems, Minneapolis, MN) was resuspended and stored according to manufacturer's instructions, and was used at a final concentration of 2 ng/ml. Constitutive active β -galactosidase expression vector, pCMV- β gal, was from Stratagene (La Jolla, CA). The MAPKAPK2 wild type, pcDNA3mycMK2 (MK2 WT), the dominant-negative kinase inactive mutant pcDNA3mycMK2 DN (MK2 DN), and the constitutive active mutant pcDNA3mycMK2 EE (MK2

EE), plasmids were generous gifts from Matthias Gaestel (Institute of Biochemistry, Medical School, Hannover, Germany) and have been described previously (Winzen et al., 1999). In MK2 DN, the conserved lysine at amino acid 76 in the ATP-binding subdomain II was replaced by arginine. Thus, MAPKAPK2 can still be phosphorylated, but it lacks kinase activity. With MK2 EE, threonine residues at 205 and 317 were mutated to aspartate, thus rendering the enzyme constitutively active. HSP27 wild type pcDNA3.1-HSP27 WT (HSP27 WT), pseudophosphorylated mutant pcDNA3.1-HSP27 3D (HSP27 3D), and nonphosphorylatable mutant pcDNA3.1-HSP27 3G (HSP27 3G) plasmids were kindly provided by Rainer Benndorf (University of Michigan, Ann Arbor, MI) and have been described previously (Schafer et al., 1999). With HSP27 3G, the activating serine residues at 15, 78, and 82 were mutated to glycine, thus precluding the enzyme from being phosphorylated and activated. For HSP27 3D, serines 15, 78, and 82 were mutated to aspartate, rendering the enzyme constitutively active. We have shown previously that MAPKAPK2 and HSP27 wild type and constitutive constructs enhance PCa cell invasion, whereas dominant-negative constructs inhibit it (Xu et al., 2006).

Cell Culture and Transfection. The origin, characteristics, and culture conditions for PC3 and PC3-M established cell lines have previously been described previously (Liu et al., 2000). Cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide with biweekly media changes, were drawn from stored stocks, replenished on a standardized periodic basis, and were routinely monitored for mycoplasma. Cell viability was routinely monitored under all experimental conditions, by counting the number of trypan blue-excluding cells under an inverted microscope using a hemocytometer; unless otherwise stated, it was not affected.

Cells were exponentially growing and nonconfluent, and were pretreated with 50 μ M genistein and 2 ng/ml TGF β (or not) as indicated. In some experiments, as indicated, cells were treated with different concentrations of genistein. Cells were transiently transfected as described previously (Liu et al., 2002; Huang et al., 2005; Xu et al., 2006). In brief, for analysis of protein phosphorylation, cells were plated into six-well plates the previous day. They were then transfected with TransIT-LT1 transfection reagent (Mirus, Madison, WI), per the manufacturer's instructions, using 2 μ g of the indicated plasmid. For measurement of matrix metalloproteinase type 2 activity by zymography, cells were transfected using LipofectAmine 2000 (Invitrogen, Carlsbad, CA), per the manufacturer's instructions, using 4 μ g of the indicated expression plasmid. For cell invasion assays, cells were transfected using LipofectAmine 2000, per the manufacturer's instructions, using 3 μ g of the indicated expression plasmid along with 1 μ g of pCMV- β -gal. The use of a constitutive active β -galactosidase vector allowed identification of transfected cells in the context of a three dimensional matrix, used in invasion assays, as described previously (Huang et al., 2005; Xu et al., 2006). For all assays, cells were treated, as indicated, 24 h after transfection.

Cell Lysis and Western Blot Analysis. Cells were lysed and Western blots performed as described previously with modifications (Liu et al., 2001; Xu et al., 2006). In brief, cells were lysed at 4°C in cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM β -glycerol phosphate) in the presence of protease inhibitors (1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 mM PMSF, all from Sigma) and phosphatase inhibitors (10 mM NaF, 1 mM orthovanadate, phosphatase inhibitor cocktails I and II, both at 1:100 dilution; all from Sigma). Resultant lysates were centrifuged at 14,000g for 30 min at 4°C, and protein concentration in the supernatant determined by Bradford dye-binding assay (Bio-Rad, Hercules, CA).

For all Western blots, equal amounts of protein were separated by SDS-PAGE on a 12% gel, under reducing conditions. Separated proteins were transferred onto 0.45- μ m nitrocellulose (Whatman Schleicher and Schuell, Keene, NH), in a wet transfer cell. Blots were blocked with 5% milk in TBST (10 mM Tris-HCl, pH 7.6, 80 mM

NaCl, and 0.1% Tween 20) for 1 h at room temperature and were probed overnight at 4°C with primary antibody diluted in TBST with 5% BSA (fraction V; Sigma). Anti-phospho-p38 MAP kinase was diluted 1:1000, anti-phospho-MAPKAPK2 was diluted 1:1000, and anti-phospho-HSP27 was diluted 1:1000. After washing, membranes were incubated for 1 h at room temperature with anti-rabbit-horse-radish peroxidase (HRP)-conjugated secondary antibody or anti-mouse-HRP-conjugated secondary antibody, and visualized by chemiluminescence, using the Enhanced Chemiluminescence Western blotting kit (GE Healthcare), per the manufacturer's instructions. Membranes were then stripped by treating with stripping buffer (100 mM β -mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min. After washing with TBST at room temperature, membranes were re-exposed (after readdition of HRP substrate to confirm removal of prior antibody) before being re-blocked and reprobed, as indicated. Specifically, for total p38 MAP kinase, diluted 1:1000; for MAPKAPK2, diluted 1:1000; for HSP27, diluted 1:1000; for GAPDH, diluted 1:5000. Each experiment was repeated one or more times, with similar results. Individual bands on Western blots were quantified using AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA), as described previously (Xu et al., 2006). All data were within the linear range of detection for each antibody used.

Zymography. Zymography assays for measurement of matrix metalloproteinase activity were performed as described previously, with modifications (Huang et al., 2005; Xu et al., 2006). In brief, 24 h after plating, equal numbers of cells were transfected with the indicated expression vector. One day later, cells were washed three times with serum-free media, incubated in serum-free media, treated as indicated, and cultured for an additional 24 h. Recounting of cells at this time point confirmed that equal numbers of viable cells were present across the different treatment conditions. Equal amounts of conditioned media were collected, centrifuged at 3000 rpm for 10 min to remove debris, and concentrated by placing in a Microcon YM-30 centrifugal filter (Millipore, Billerica, MA), and spinning at 14,000g for 12 min. Equal amounts of concentrated media were mixed with 2× sample buffer (125 mM Tris, pH 6.8, 1% SDS, 0.002% bromophenol blue, and 10% glycerol), incubated 15 min at room temperature, and then separated on a 9% SDS polyacrylamide gel containing 1 mg/ml gelatin, under nonreducing conditions. Gels were washed with 2.5% Triton X-100 for 30 min and incubated for up to 48 h at 37°C in 20 mM glycine, pH 8.3, 10 mM CaCl_2 , and 1 μM ZnCl_2 . Gels were then stained with 0.5% Coomassie Brilliant Blue G solution, containing 10% acetic acid and 20% methanol, for 30 min, destained with 10% acetic acid and 20%

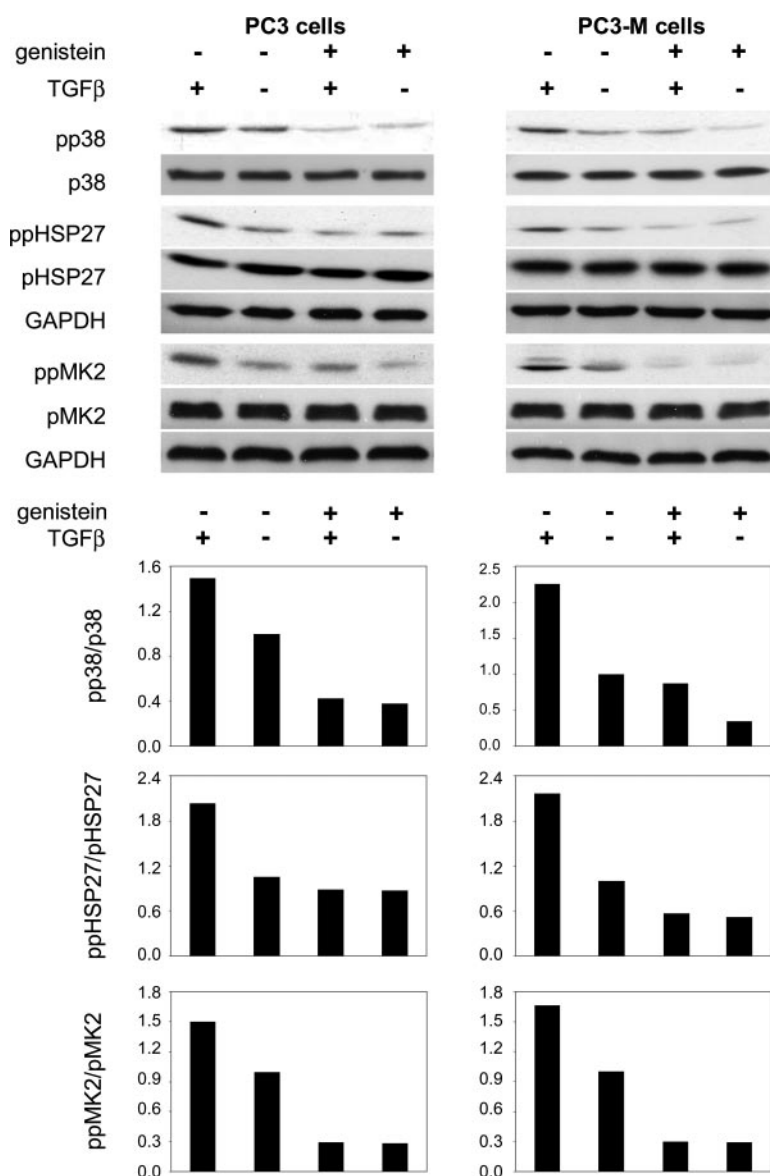


Fig. 1. Genistein inhibits TGF β -induced activation of MAPKAPK2 and HSP27. One day after plating, PC3 and PC3-M cells were changed to serum-free media, and treated with or without genistein for 23 h. After being treated with or without TGF β for 10 min (for MAPKAPK2) or 30 min (for p38 MAP kinase and HSP27), cells were lysed and equal amounts of protein were separated and probed for phospho-p38 MAP kinase (pp38), phospho-MAPKAPK2 (ppMK2), and phospho-HSP27 (ppHSP27) by Western blot, as described under *Materials and Methods*. After stripping, the blot was re-probed for total p38 MAP kinase (p38), total MAPKAPK2 (pMK2), total HSP27 (pHSP27), or GAPDH. Individual bands from a single experiment were quantitated, expressed as the indicated ratio of phospho-protein to total protein, and resultant data graphed on the bottom. Similar results were seen in multiple replicate experiments, performed at separate times.

methanol, and visualized. Individual bands were quantified using AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA).

Cell Invasion Assays. Cell invasion assays were performed as described previously, with modifications (Liu et al., 2002; Huang et al., 2005; Xu et al., 2006). In brief, 24 h after plating, cells were transfected with the indicated expression vector, along with a constitutive active β -galactosidase expression vector, thus allowing detection of transfected cells. One day later, cells were treated as indicated, detached by treatment with Trypsin/EDTA, washed, resuspended in RPMI 1640 cell culture medium (Invitrogen) with 0.1% BSA, and 52 μ l of cell suspension were placed into the upper chamber of a 48-well Boyden chamber unit (i.e., 10^4 cells/well). Cells were allowed to migrate for 10 h through a Nuclepore Track-Etch membrane (Whatman, Clifton, NJ), which contained 8- μ m pores and was coated with gelatin, toward serum-free NIH-3T3 conditioned medium present in the lower chamber. Membranes were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 min at room temperature, rinsed with PBS, and β -galactosidase was detected using an in situ β -galactosidase staining kit (Stratagene) according to the manufacturer's instructions. Cells were then stained with solution 1 (i.e., xanthane dye) using Diff-Quick cell-staining kit (Dade Behring AG, Duding, Switzerland). Membranes were mounted onto slides using Permount (Fisher Scientific, Hampton, NH). Using predetermined field coordinates, the numbers of invading and noninvading cells were then counted in each of five high-power fields (i.e., $100\times$) for a given well, times four wells for each treatment condition ($n = 4$). All statistical tests of invasion were two-sided, and changes were considered statistically significant only for t test p values of 0.05 or less. All experiments were repeated at least once, at a separate time, in quadruplicate.

Results

Genistein Inhibits TGF β -Induced Activation of MAPKAPK2 and HSP27. To evaluate genistein's effects upon MAPKAPK2 and HSP27 activation, we first sought to determine whether it could block their phosphorylation. Studies were carried out with PC3 and PC3-M human PCa cells, which have been extensively characterized by us for their motility and their response to genistein (Bergan et al., 1996; Kyle et al., 1997; Liu et al., 2000, 2001, 2002; Hayes et al., 2003; Huang et al., 2005; Xu et al., 2006). PC3-M cells represent an aggressive metastatic variant. Cells were pretreated with genistein (or not), then treated with TGF β (or not), and levels of phospho-MAPKAPK2 or phospho-HSP27 were measured by Western blotting (Fig. 1). In the absence of genistein, TGF β increased phosphorylation of p38 MAP kinase, MAPKAPK2, and HSP27. In contrast, pretreatment with genistein almost completely abrogated TGF β -mediated increases in protein phosphorylation. Similar results were seen in both cell lines tested. In cells pretreated with genistein before TGF β , levels of phospho-MAPKAPK2 decreased by 5.0- and 5.4-fold in PC3 and PC3-M cells, respectively, compared with cells treated with TGF β alone. Phospho-HSP27 levels were decreased by genistein by 2.4- and 3.6-fold, respectively, in PC3 and PC3-M cells. Thus, genistein inhibited TGF β -mediated activation of p38 MAP kinase, MAPKAPK2, and HSP27 in human PCa cells.

Genistein's Inhibitory Effect upon TGF β -Induced Activation of HSP27 Is Dependent on MAPKAPK2. HSP27 can be phosphorylated by kinases in addition to MAP-

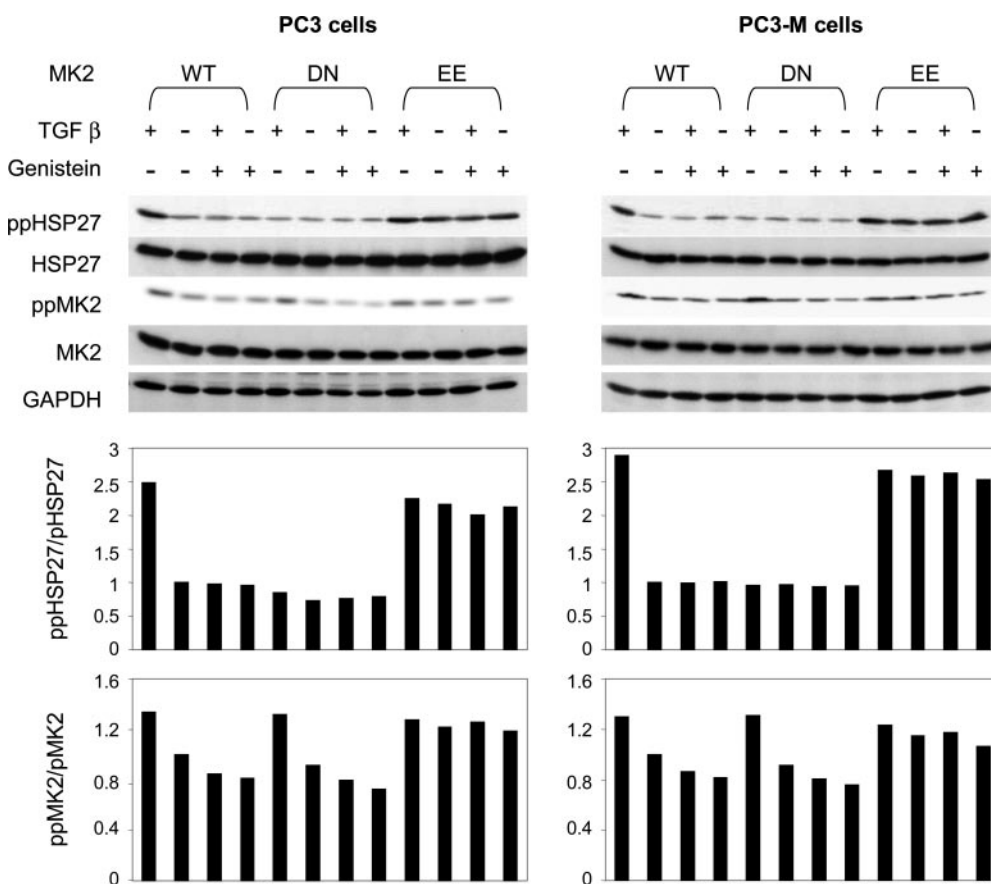


Fig. 2. Inhibition of HSP27 activation by genistein is through MAPKAPK2. PC3 and PC3-M cells were transfected with MK2 WT, MK2 DN, or MK2 EE. Twenty-four hours after transfection, cells were changed to serum-free media. After being treated with or without genistein for 23 h, TGF β was added for 15 min before cells were lysed. Phospho-MAPKAPK2 (ppMK2), total MAPKAPK2 (pMK2), phospho-HSP27 (ppHSP27), total HSP27 (pHSP27), and GAPDH were measured by Western blot, and resultant bands from a single experiment were quantitated, as described in Fig. 1. Similar results were seen in a separate experiment, performed at a different time.

KAPK2 (Landry et al., 1992; Ludwig et al., 1996; Maizels et al., 1998; Butt et al., 2001; Doppler et al., 2005). If genistein's effects upon HSP27 phosphorylation were dependent solely on MAPKAPK2, then constitutively active MAPKAPK2 should rescue cells from genistein's inhibitory effects. Conversely, if genistein's effect upon HSP27 were mediated through other kinases, or if genistein were acting directly on HSP27, then it would also inhibit the phosphorylation of HSP27, even in cells transfected with constitutive active MAPKAPK2. PC3 and PC3-M cells were therefore transfected with the MK2 WT, MK2 DN, or MK2 EE MAPKAPK2 constructs, and phosphorylation of HSP27 was measured. As shown in Fig. 2, levels of phospho-HSP27 were high in EE transfected cells and, importantly, were not affected by treatment with either TGF β or genistein. In contrast, phospho-HSP27 levels were low in DN-transfected cells and were similarly not affected by either TGF β or genistein. In WT transfected cells, TGF β retained its stimulatory effect and genistein retained its inhibitory effect. These findings demonstrate that genistein's inhibitory effect upon TGF β -induced activation of HSP27 was dependent on MAPKAPK2.

Genistein Inhibits TGF β -Regulated Increases of MMP-2 Activity and Cell Invasion through Inhibition of MAPKAPK2-HSP27 Pathway. We have recently shown that p38 MAP kinase activates MAPKAPK2, that MAPKAPK2 in turn activates HSP27, and that HSP27 regulates

MMP-2 and cell invasion (Xu et al., 2006). Furthermore, we have shown that genistein inhibits MMP-2 and cell invasion (Huang et al., 2005). It therefore stands to reason that MAPKAPK2 and HSP27 may play an important role in mediating genistein's effects upon these processes. To examine this, PC3 and PC3-M cells were first transiently transfected with MK2 WT, MK2 DN, or MK2 EE or HSP27 WT, HSP27 3D, or HSP27 3G. Cells were then pretreated (or not) with genistein followed by treatment (or not) with TGF β , and MMP-2 activity was measured by zymography (Figs. 3, a and b). In wild-type cells, TGF β increased MMP-2 activity, and pretreatment with genistein abrogated TGF β -mediated increases. In contrast, in MK2 DN or HSP27 3G and MK2 EE or HSP27 3D, neither TGF β nor genistein had any effect upon MMP-2 activity. For MK2 DN and HSP27 3G, activity remained low, whereas for MK2 EE or HSP27 3D, activity remained high. Similar findings were observed in both cell lines tested.

The functional significance of genistein's dependence upon the MAPKAPK2-HSP27 pathway was further evaluated by measuring effects upon cell invasion (Fig. 4, a and b). TGF β increased cell invasion in wild-type cells, whereas pretreatment with genistein abrogated TGF β -mediated increases in invasion. Genistein's effects upon MAPKAPK2 and HSP27 wild-type experiments together, pretreatment with genistein before TGF β decreased invasion on average to 56%

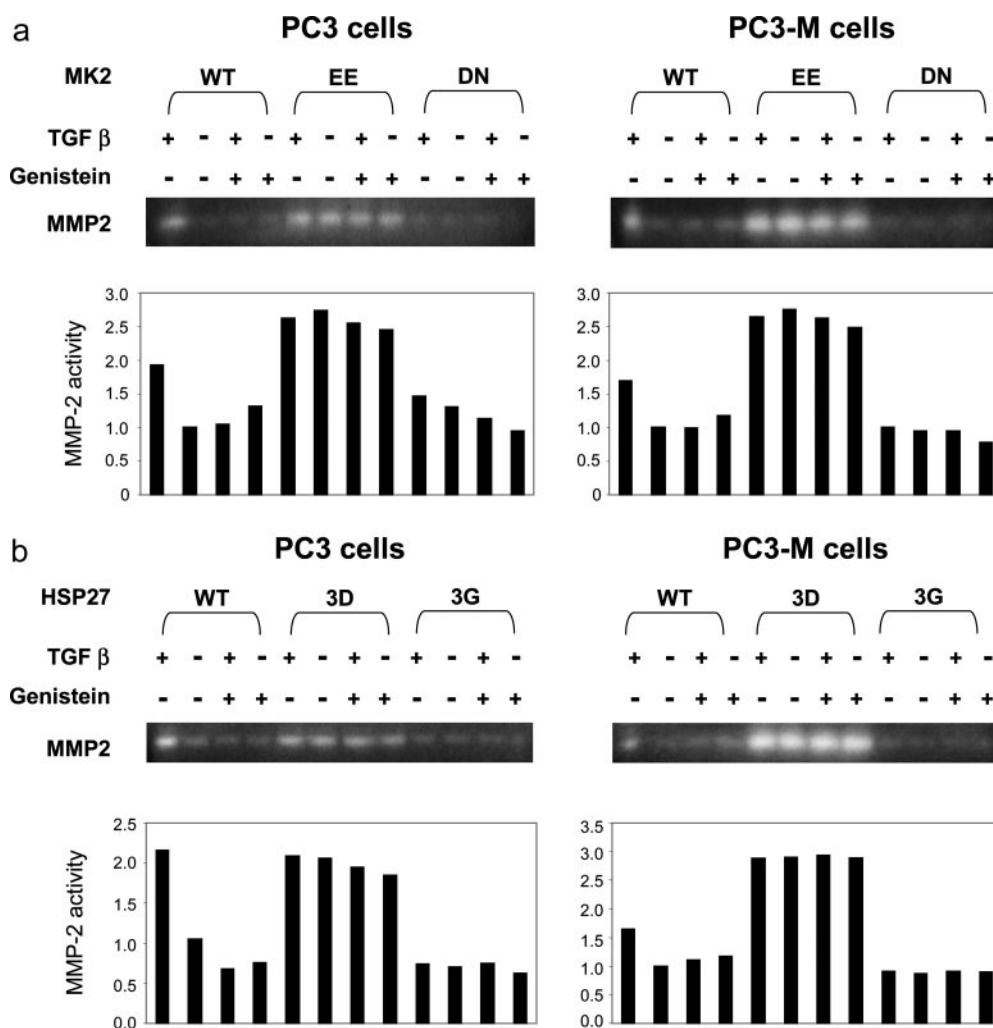


Fig. 3. Genistein's effects on MMP-2 activity are dependent upon MAPKAPK2 or HSP27. PC-3 and PC3-M cells were transfected with MK2 WT, MK2 EE, or MK2 DN (a) or with HSP27 WT, HSP27 3D, or HSP27 3G (b). One day after transfection, cells were changed to serum-free media, pretreated with genistein (or not) for 1 h, then treated with TGF β (or not) for 23 h, and conditioned media were collected. MMP-2 activity was measured by zymography, as described under *Materials and Methods*. Individual bands from a single experiment were quantitated, expressed as the amount of MMP-2 activity relative to wild-type cells (not treated with either TGF β or genistein), and resultant data graphed at the bottom. Similar results were obtained in multiple separate experiments, performed at separate times.

and 53% of levels seen compared with PC3 and PC3-M cells, respectively, treated with TGF β alone. In MK2 DN or HSP27 3G cells, baseline levels of invasion were at or slightly below those of baseline levels in wild-type cells. It is noteworthy that with MK2 DN or HSP27 3G cells, levels of invasion after TGF β treatment were significantly below those seen in wild-type cells after TGF β treatment. In addition, treatment with genistein did not further decrease invasion in MK2 DN or HSP27 3G cells. With MK2 EE or HSP27 3D cells, baseline levels of invasion were high, approaching levels seen after TGF β treatment of wild-type cells. Neither TGF β nor genistein had additional effects. Finally, we have shown previously that MAPKAPK2 activates HSP27 (Xu et al., 2006), and we show in Fig. 1 that genistein blocks activation of MAPKAPK2. The current experiment therefore demonstrates that HSP27 3D can rescue cells in the face of inactive MAPKAPK2. For all treatment categories, similar findings were seen in both cell lines tested. These findings demonstrate that genistein-mediated inhibition of TGF β 's effects

upon MMP-2 activation and cell invasion are dependent upon the MAPKAPK2-HSP27 pathway.

Genistein Inhibits TGF β -Mediated Activation of HSP27 at Physiological Concentration. Genistein is found in soy. Adlercreutz et al. (1993) demonstrated that the concentration of free genistein in the blood of soy-consuming Japanese men ranged between 3 and 15 nM. We conducted a prospective phase I study of genistein in an older cohort of men with PCa, thereby characterizing its pharmacokinetics (Takimoto et al., 2003). We demonstrated that 90% of circulating genistein was inactivated by first-pass liver metabolism (primarily by glucuronidation and sulfation) and thus confirmed prior findings by Adlercreutz et al. (1993). It is noteworthy that we demonstrated that with prospective dosing, blood concentrations of free genistein were in the 10 to 100 nM range. We have previously demonstrated that genistein's effects upon PCa cell motility are time- and concentration-dependent (Bergan et al., 1996; Huang et al., 2005). In particular, after a 24-h treatment period, 50 μ M

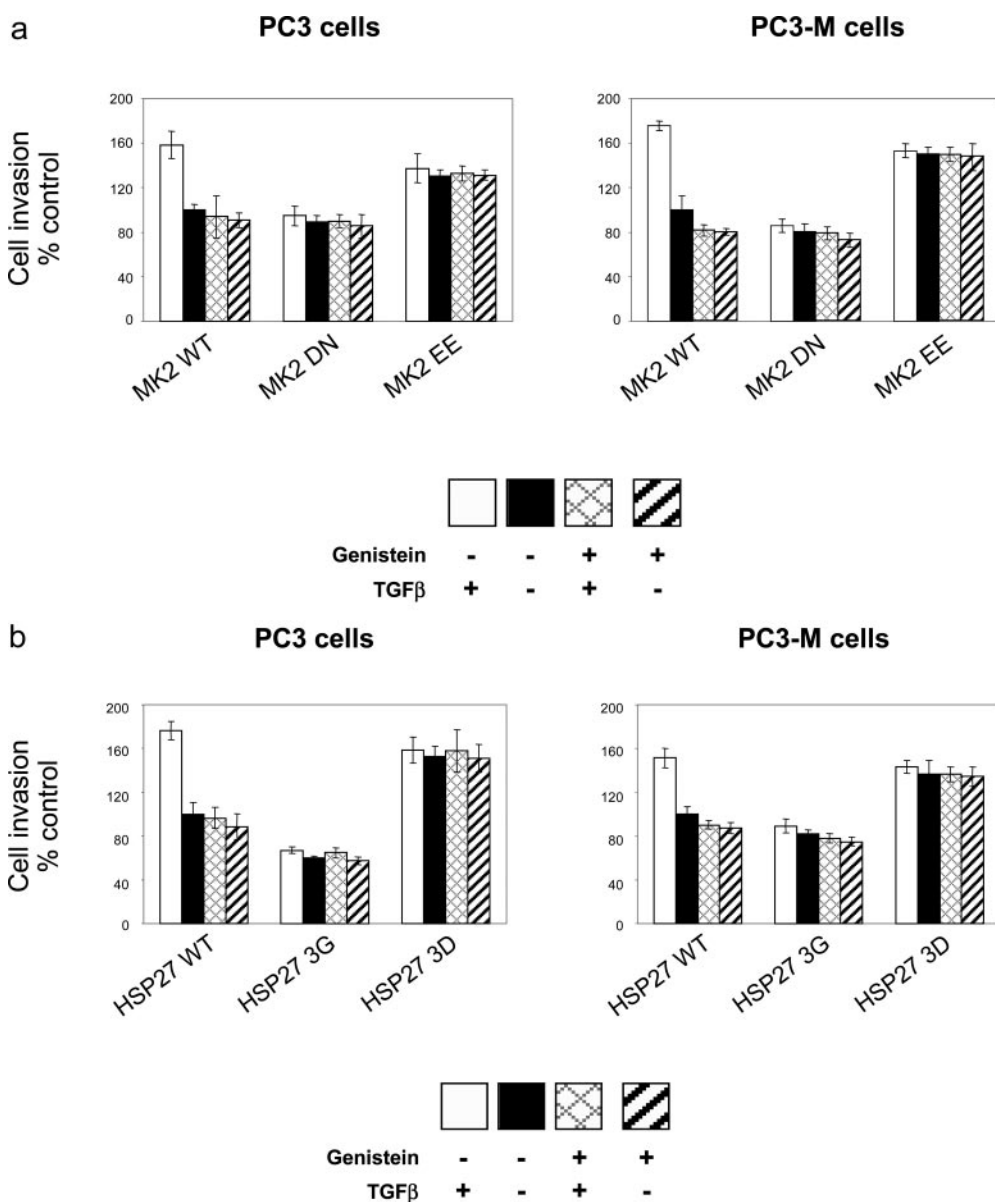


Fig. 4. Genistein's effects on cell invasion are dependent upon MAPKAPK2 or HSP27. PC-3 and PC3-M cells were transfected with MK2 WT, MK2 EE, or MK2 DN (a) or with HSP27 WT, HSP27 3D, or HSP27 3G (b). One day after transfection, cells were pretreated with genistein (or not) for 1 h, then treated with TGF β (or not) for 23 h, and cell invasion was measured. Cell invasion is depicted as the percentage of invading cells, relative to untreated control cells, and is the mean \pm S.E.M. ($n = 4$) of a single experiment. Similar results were obtained in a separate experiment performed at a separate time, also $n = 4$. Cell transfection and invasion assays were performed as described under *Materials and Methods*. The two-sided t test p value was below 0.05 for the following comparisons, for both cell lines, and for both MK2 and HSP27 experiments: TGF β -treated versus not for wild-type cells; TGF β -treated wild-type versus all treatment categories of dominant-negative cells; wild-type (not treated with TGF β) versus all treatment categories of MK2 EE or HSP27 3D cells.

genistein will decrease cell invasion. Therefore, this concentration and treatment time was used in the above studies. However, we have previously shown that if treatment time is extended to 3 days, then anti-invasive efficacy can be observed with genistein concentrations in the 10 to 100 nM range (Huang et al., 2005).

If the MAPKAPK2-HSP27 pathway was in fact therapeutically relevant, then a response to low to midnanomolar concentrations of genistein should be evident. This was shown to be the case by first transfecting PC3 and PC3-M cells with either wild type HSP27 (WT) or empty vector (VC). Cells were then treated with genistein for 3 days at concentrations ranging from 0.01 to 10 μ M and treated with TGF β for the final 60 min, and then levels of phospho-HSP27 were measured (Fig. 5). Genistein did not affect cell viability under these conditions. With VC cells, phospho-HSP27 levels began to decrease in both cell lines tested with concentrations of genistein in the 10 to 100 nM range. With higher genistein concentrations, phospho-HSP27 levels decreased to levels approximating half those seen in untreated VC cells. In WT cells, decreases in phospho-HSP27 were also observed but were blunted. In particular, the percentage decrease with the highest dose of genistein was less. Furthermore, in PC3 cells, decreases were not observed until concentrations of 1 μ M were used. Thus, these findings demonstrate that HSP27 is responsive to physiological concentrations of genistein. However, they also indicate that up-regulation of HSP27 can partially negate genistein's effects.

Discussion

We have demonstrated previously that genistein inhibits TGF β -mediated activation of p38 MAP kinase, inhibition of MMP-2, and PCa cell invasion (Huang et al., 2005). We recently discovered that MAPKAPK2 and HSP27 are down-

stream effectors of p38 MAP kinase and that they were important regulators of MMP-2 activity and cell invasion in response to TGF β (Xu et al., 2006). Although both MAPKAPK2 and HSP27 are downstream of p38 MAP kinase, each can be activated by kinases other than p38 MAP kinase. In addition, increased HSP27 has been associated with prostate cancer, and seems to contribute to carcinogenic progression (Cornford et al., 2000; Rocchi et al., 2005). This raised the possibility that elevated levels of HSP27 could overcome and/or bypass any upstream inhibitory effects of genistein.

An understanding of the molecular mechanisms that underlie the role of genistein in antagonizing TGF β 's effects upon MMP-2 activation and cell invasion is important. It is necessary to optimize the design of trials seeking to test genistein's antimetastatic efficacy. It is also necessary to optimize our understanding of the therapeutic potential of this pathway for future agents. Finally, it is necessary to understand the effect of therapeutic manipulation in the face of signaling pathway alterations that occur coincident with carcinogenic progression.

This study therefore sought to elucidate genistein's effects upon MAPKAPK2 and HSP27. We propose the schema depicted in Fig. 6. We show for the first time that TGF β -regulated activation of the MAPKAPK2-HSP27 pathway was inhibited by genistein. By going on to demonstrate that this led to inhibition of MMP-2 activation and cell invasion, we demonstrated the functional significance of therapeutically modulating this pathway.

The broader relevance of this to human PCa was supported by the fact that we demonstrated efficacy in PC3 cells, as well as in metastatic variant PC3-M cells. This suggests that genistein's antimotility efficacy is retained in the face of an altered motility phenotype and demonstrates that findings are not particular to a specific cell line. An exhaustive series

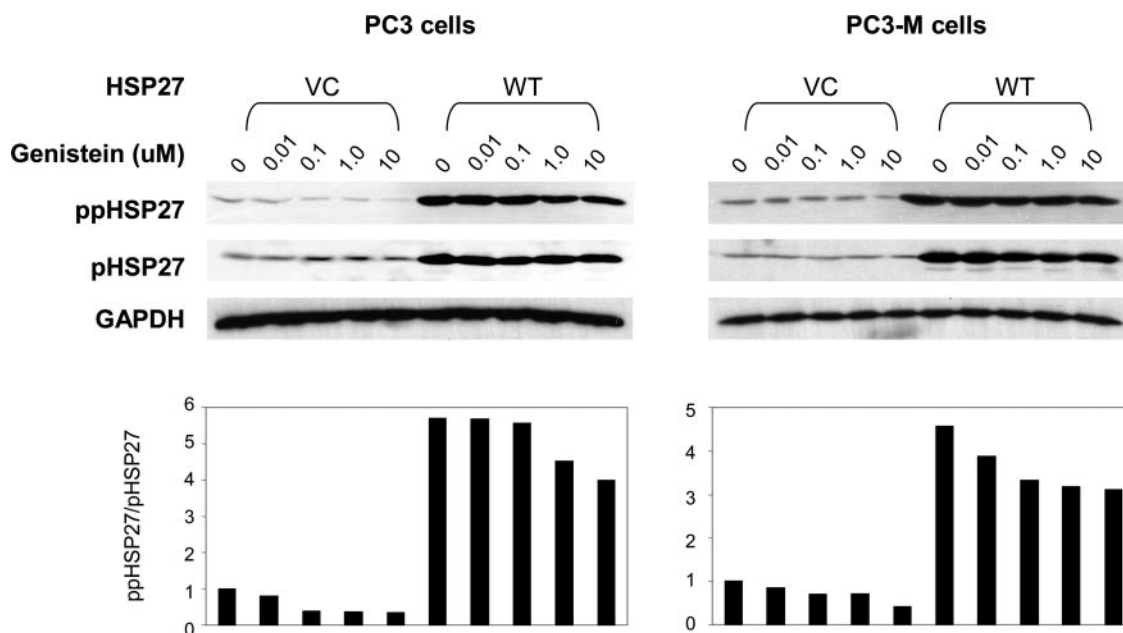


Fig. 5. Genistein inhibits TGF β -mediated activation HSP27 at physiological concentrations. PC3 and PC3-M cells were transfected with either HSP27 wild-type (WT) or empty vector for control (VC). One day after replating, cells were treated (or not) with the indicated concentrations of genistein for 72 h. Cells were then treated with TGF β for 60 min, lysed, and equal amounts of clarified protein were probed for phospho-HSP27 (ppHSP27), total HSP27 (pHSP27), or GAPDH by Western blot. Resultant bands from a single experiment were quantitated, expressed as the indicated ratio ppHSP27/Phsp27, and data depicted graphically in the lower panels, all as described in Fig. 1. Similar results were seen in multiple replicate experiments performed at separate times.

of studies by us has characterized the different motility phenotypes of these two cell lines (Bergan et al., 1996; Kyle et al., 1997; Liu et al., 2000, 2001, 2002; Hayes et al., 2003; Huang et al., 2005; Xu et al., 2006). Of particular relevance is that we have recently shown that PC3-M cells contain higher levels of HSP27 than do PC3 cells (Xu et al., 2006). Others have implicated HSP27 in PCa progression, and their analysis of clinical samples has shown that HSP27 is increased in human PCa (Cornford et al., 2000; Rocchi et al., 2005). Finally, the broader relevance of the current findings is further supported by our prior work, in which we demonstrated that genistein decreased MMP-2 activity in six different human prostate cell lines (Huang et al., 2005). Cell lines in this panel spanned the spectrum of prostatic carcinogenesis and ranged from normal prostate epithelial cells to highly aggressive metastatic variant cells (Liu et al., 2001).

Genistein's potential as an effective therapeutic agent was highlighted by the fact that it retained efficacy even in the face of exogenous TGF β treatment in the context of overexpressed MAPKAPK2 or HSP27. As a corollary to efficacy, pathway specificity was also addressed and demonstrated. In particular, specific reliance upon the MAPKAPK2-HSP27 pathway was demonstrated and was done so using complementary approaches. That is, genistein's effects were shown to be abrogated when pathway responsiveness was negated by either constitutive activation or by use of dominant-negative kinase inactive constructs of MK2 and nonphosphorylatable constructs of HSP27.

However, these later findings highlight a potential limitation in therapeutic efficacy. That is, if pathway activation occurs during prostatic carcinogenesis, genistein's efficacy could be curtailed. In fact, increased HSP27 protein expression has been reported during prostate cancer progression (Cornford et al., 2000; Rocchi et al., 2005). Therefore, we went on to evaluate the efficacy of lower genistein concentrations in the context of high levels of HSP27. Two important conclusions came from this later series of studies. The first is that low to midnanomolar concentrations of genistein will

block activation of HSP27. These concentrations emulate blood concentrations of free genistein attained in men after oral dosing (Takimoto et al., 2003) and are thus physiologically relevant. The second conclusion was that in the context of a limiting amount of genistein, high levels of wild-type HSP27 could negate its efficacy, at least in part. Thus, those who have high HSP27 levels may require higher doses of genistein.

In summary, current findings represent an extension of our prior findings. They demonstrate for the first time that genistein antagonizes the effects of TGF β upon MMP-2 and cell invasion in human PCa by blocking MAPKAPK2-HSP27 pathway signaling. These findings support the notion that genistein has potential as an antimetastatic therapeutic agent in PCa. They also demonstrate that genistein's efficacy may be limited in persons with elevated expression of MAPKAPK2-HSP27 pathway members. However, they further suggest that this could be overcome by increasing the dose of genistein.

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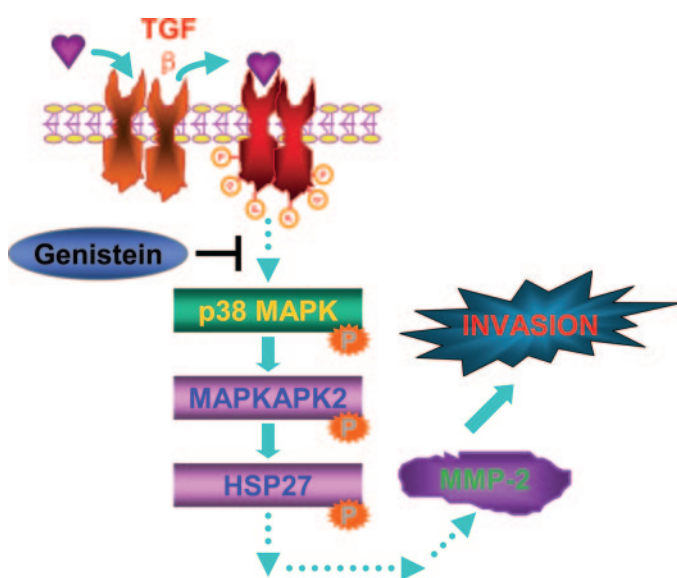


Fig. 6. Proposed schema for genistein's mechanism of action. Genistein antagonizes TGF β 's effects upon MMP-2 activation and cell invasion in human prostate cancer. These effects are mediated through the MAPKAPK2-HSP27 pathway.

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