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Rapamycin induces Smad activity in prostate cancer cell lines

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Abstract Rapamycin inhibits the FK506-binding protein 12 (FKBP12)/mammalian target of rapamycin (mTOR) complex and causes cell cycle arrest in G1. The precise mechanism of growth inhibition by rapamycin is only partly understood. Rapamycin led to growth inhibition in the human prostate cancer cell lines LNCaP and PC3 cells after 72 h, ID50: 93 and 50 nM, respectively. Filter cDNA array analysis showed down-regulation by more than 0.75× by rapamycin in PC3 cells and LNCaP cells of the following genes: follistatin, eukaryotic initiation factor-4E (eIF4E), glucose-6-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH)-A, ATP synthase, heat shock protein (HSP)-1. Upregulation by more than 1.5 × was found for: bone morphogenetic protein (BMP)-4, FKBP12, carcinoma embryonic antigen (CEA) precursor, eukaryotic initiation factor (eIF)-3 p36 subunit, latent transforming growth factor (TGF) beta binding protein (LTBP)1. Rapamycin induced BMP4 and reduced follistatin expression in PC3 cells. This resulted in a dose-dependent nuclear expression of Smad4 and activated the SBE4 Smad-reporter, indicating activation of TGFbeta/BMP signaling. Combining rapamycin with PI3K inhibition (LY294002) increased growth inhibition. These findings illustrate that Smad signaling plays a role in the anticancer effects of rapamycin and show that combination with PI3K inhibition improves growth inhibition.

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Introduction

Prostate cancer (PCa) is the most common nonskin cancer in men in the Western world (SEER data, *seer.cancer.gov*). Although the majority of advanced tumors responds to initial androgen ablation therapy, hormone-refractory cancers are associated with a 5-year survival of less than 25%, and 80% of patients die of disease [21]. Chemotherapeutic treatment modalities show only a modest effect, with objective response rates in 10%–20% of cases [2]. New developments in anticancer treatment can be found in the inhibition of signal transduction pathways. Many tumor cells are dependent on altered growth factor signal transduction providing growth advantage [12]. For example, V12 mutations in the H-*ras* gene render its gene product continuously active, with subsequent activation of the mitogen-activated protein kinase (MAPK) pathway [17], whereas a phosphatase and tensin homologue deleted on chromosome 10 (PTEN) function loss results in an overactive phosphoinositide 3-kinase (PI3K)/serine-threonine kinase (AKT) pathway [15]. Both pathways have been found to promote prostate cancer [9, 14].

The macrolide rapamycin inhibits signal transduction through inhibition of the mammalian target of rapamycin/FKBP12-rapamycin-associated protein/FK506-binding protein 12 (mTOR/FRAP/FKBP12) complex and is used as an immunosuppressive agent. The mTOR complex is involved in several aspects of cell survival and activated through protein kinase B/AKT phosphorylation. mTOR activation results in an induction of basal $p70^{S6\ kinase}$ activity and phosphorylation of 4E binding protein 1 (4E-BP1) [23]. An increase in 4E-BP1 phosphorylation is associated with a decreased binding of 4E-BP1 to the eukaryotic initiation factor 4E (eIF-4E),

rendering eIF-4E available for binding eIF-G, resulting in the initiation of cap-dependent translation [23]. Overexpression of eIF-4E is common in cancers of the head and neck, breast, and prostate, and associated with vascular endothelial growth factor (VEGF) overexpression [6]. These important functions of the mTOR/FRAP/FKBP12 complex make rapamycin an interesting agent for cancer-specific growth inhibition [20]. A derivative of rapamycin, CCI-779, is currently being evaluated as an anticancer drug in phase-I clinical trials [1].

Rapamycin was found to inhibit induction of hypoxia-inducible factor 1-alpha (HIF1alpha). HIF1alpha is normally overexpressed under hypoxic conditions and induces among other things the expression of vascular endothelial growth factor. Moreover, HIF1alpha was shown to be overexpressed in many prostate cancers [29]. Since many prostate cancers were shown to harbor hypoxic tumor areas, rapamycin is an interesting agent to counteract the growth-promoting functions of HIF1alpha.

To evaluate the effects of rapamycin on human PCa cell lines, we performed analysis of growth inhibition in PC3 and LNCaP. Moreover, the effects on changes in the transcriptome using cDNA array technology were studied. Data from these analyses show dose-dependent growth inhibition of PCa cell lines by rapamycin monotherapy in vitro. Gene expression changes assessed by cDNA array technology showed more pronounced changes in PC3 than in LNCaP. The induction of bone morphogenetic protein 4 (BMP4) and suppression of follistatin expression by rapamycin as shown by array and confirmed by immunoblot analysis suggest a role of transforming growth factor (TGF) signaling in growth inhibition.

Materials and methods

Cell cultures. LNCaP and PC3 cells were grown to 90% confluency in T75 flasks in RPMI 1640 medium containing 10% fetal bovine serum (FBS) in 20% oxygen, 5% CO_2. Subsequently, the supernatant was aspirated, the cells were washed once with phosphate-buffered saline (PBS) solution, and 10 ml of 10% FBS containing RPMI 1640 medium was added. Cells were grown for 24 h. The medium was aspirated, and RPMI 1640 medium containing 10% FBS and a dose range of rapamycin (0.1, 10, 20, 50, 100, 500, 1000 nM) was added. Cells were grown for 8 or 24 h and harvested for RNA or protein extraction. Hypoxia experiments were done in 1% oxygen, 5% CO_2, 94% nitrogen in hypoxia chambers (Billups-Rothenburg, Del Mar, CA, USA).

Growth inhibition assays. To study the growth inhibition in vitro, 10^4 cells per well were plated in 96-well plates, and after 24 h, rapamycin (Sigma, St Louis, MI, USA) was added in different concentrations for different time intervals (8, 24, 48, 72 h). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Trevigen, Gaithersburg, MD) was performed according to the manufacturer's protocol. Experiments were performed in 10% FBS and normoxic (20% O_2) and hypoxic (1% O_2) conditions. Plates were read at 570 nm, and optical density values were normalized to baseline values. The following agents were used in combination with rapamycin: LY294002 (Sigma), 2'-amino-3'-methoxyflavone (PD98059), *N*-(2(S)-[2(*R*)-amino-3-mercaptopropylaminol-3-methylbutyl)-Phe-Met-OH, a farnesyl transferase inhibitor (FTI) (Alexis, San Diego, CA, USA).

RNA and protein extraction. For RNA extraction, cells were harvested using trypsin solution, resuspended in PBS, centrifuged, and suspended in lysis buffer. The RNeasy kit (QIAGEN, Valencia, CA, USA) was used for RNA extraction. Samples were dissolved in distilled water and stored at –80°C. For protein extraction, the previously published protocol was used [29]. The BCA protein assay kit (Pierce, Rockford, IL, USA) was used for quantification of the total protein content and expressed in μg per ml. RNA content was assessed using the orcinol assay.

Filter array analysis. The filter arrays used (GF211, Research Genetics, Huntsville, AL, USA) contained 4113 named genes. Filters were probed using ^{33}P-labeled cDNA according to the manufacturer's protocol. Filters were read using a phosphoimager. Images were analyzed using the Pathways 2.01 software (Research Genetics). To prevent background differences from interfering with low-level gene expression, the undetectable level was set at 2000 units normalized value as calculated by the Pathways 2.01 software. Each comparison was performed twice.

cDNA probes and Northern analysis. The following cDNAs were obtained by digestion using the appropriate enzymes from the bacterial clones (IMAGE cDNA ID number): ATP binding protein (488303), BMP4 (797048), and follistatin (434768) (Research Genetics). Northern blotting was performed using 1% denaturing agarose gel (Ambion, Austin, TX, USA). RNA was loaded at 15 μg per well in 15 μL loading dye and run for 3 h at 70 volts. The gel was washed in 10×SSC (sodium chloride/sodium citrate) and blotted onto nitrocellulose paper overnight. The probes were labeled with α-dCTP ^{32}P using a random primer kit (Rediprime II, Amersham, Piscataway, NJ, USA). After prehybridization in Quickhyb (Stratagene, La Jolla, CA, USA) for 1 h, 1.25e6 CPM/ml of the labeled probe was used for hybridization for 1 h. Blots were washed in 0.1×SSC/0.1% sodium dodecyl sulfate (SDS) at 60°C and exposed to X-omat AR film (Kodak, Rochester, NY, USA) overnight.

Immunoblot assays. Protein samples were dissolved in SDS loading buffer and denaturated by boiling for 5 min. A 7% SDS-polyacrylamide gel electrophoresis (PAGE) stacked gel was cast, and samples were run for 2 h at 150 volts. Blotting was performed overnight on nitrocellulose paper. Antibodies against the following proteins were used for Western analysis: BMP4, Smad4 (Santa Cruz, CA, USA), follistatin (R&D, Minneapolis, MN, USA), and HIF1a (Novus, Littleton, CO, USA).

Smad-report assays. The Smad-binding element containing luciferase reporter (pGL-SBE4-luc) was used for the assessment of Smad transactivation [13]. To control for transfection efficacy, a co-transfection with a CMV-beta galactosidase-containing plasmid was used and beta-galactosidase activity assessed using densitometry at 425 nm after incubation of lyzed cells with *ortho*-nitrophenyl-*b*-D-galactopyranoside (ONPG) -$MgCl_2$ sodium phosphate solution. Since rapamycin influences the translation of certain proteins, a CMV-luc control plasmid was added to the reaction to control for translatory changes caused by rapamycin. For both control plasmids, as well as the pGL-SBE4-luc reporter, 100 ng of DNA were added to 10e4 cells cultured overnight in a 96-well plate. For reporter assays, cells were incubated with rapamycin for 48 h at different doses.

Statistical analysis. Pearson correlation values were used to compare expression levels of the various mRNAs. Each array experiment was performed in duplicate. Growth inhibition analyses were performed twice with five wells for each drug dose or combination.

Results

In vitro experiments with the cell lines PC3 and LNCaP showed growth inhibition with ID_{50} of 93 and 56 nM rapamycin, respectively (Fig. 1). LNCaP was significantly less sensitive to rapamycin compared with PC3.

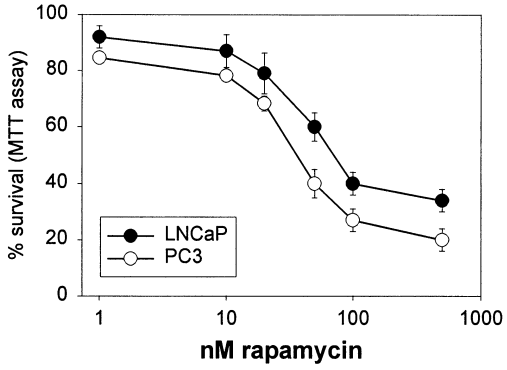


Fig. 1 Dose-response curves for LNCaP and PC3 cells after 72 h exposure to different doses of rapamycin

Although a trend existed towards increased growth inhibition by rapamycin under hypoxic conditions as compared with normoxic cultures, these differences were not statistically significant (data not shown).

Using filter array analyses a strong positive correlation existed for overall expression differences after 10 nM and 20 nM rapamycin in PC3 cells ($r = 0.587$, $p = 0.001$) and LNCaP cells ($r = 0.277$, $p = 0.001$). When relative expression for the same genes between LNCaP and PC3 cells were compared, the correlation was negative with the highest expression levels in PC3 cells ($r = -0.184$, $p = 0.001$), indicating that overall expression patterns as a response to rapamycin differed between the two cell lines. Several genes were similarly regulated on the mRNA level in both LNCaP and PC3 cells (Table 1). Glucose-6-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase A (LDH-A) were both down-regulated by rapamycin and up-regulated by hypoxia as described earlier [25, 28].

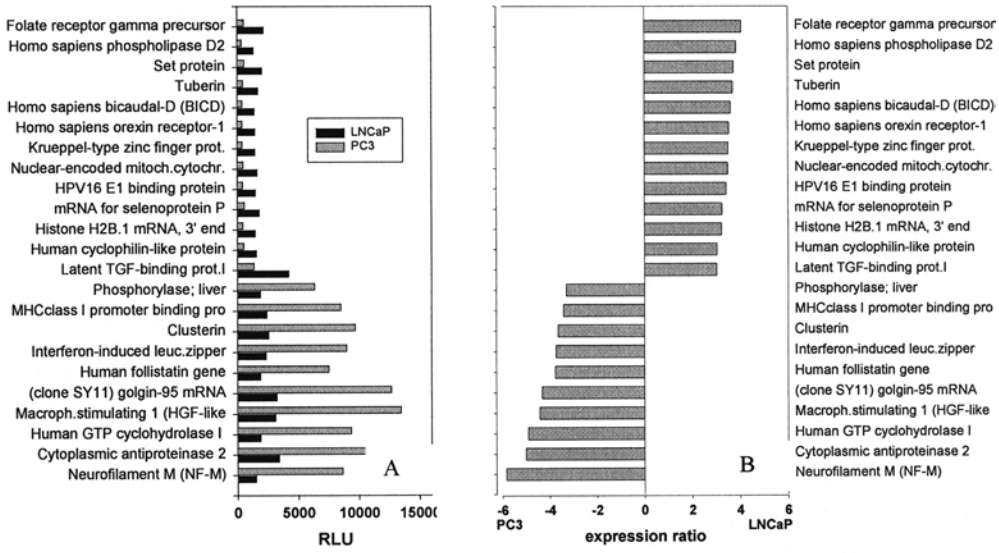
Figure 2a shows the genes expressed 3 × less or more in LNCaP cells compared to PC3 cells using filter array analysis. Ratios were calculated after correcting for loading and background (Fig. 2b). Filter array images of an area of the filter array illustrating overexpression of latent transforming growth factor beta-binding protein 1, follistatin and macrophage stimulating factor (hepatocyte growth factor-like) in PC3 cells compared to LNCaP cells are shown in Fig. 2c. Hence, of the differently expressed genes, two were associated with TGFbeta signaling, whereas the majority of genes related to this signaling pathway were not differently expressed between LNCaP and PC3 cells (Table 2).

The changes in expression of bone morphogenetic protein 4 (BMP4) and the inhibitor follistatin were confirmed by northern blotting in PC3 cells. Hypoxia reduced follistatin mRNA expression in both LNCaP and PC3 cells (Fig. 3a). After 24 h of rapamycin exposure, follistatin expression decreased in both normoxic and hypoxic PC3 cells (Fig. 3b). BMP4 mRNA expression decreased under hypoxia and was increased by rapamycin at both 8 h and 24 h (Fig. 3b). The filter cDNA arrays showed mRNA expression changes of several other TGFbeta superfamily members (Table 2). Rapamycin-induced down-regulation was the strongest for follistatin mRNA in PC3 (0.61), whereas the strongest overexpression was found for BMP4 (1.71) and latent TGF binding protein 1 (LTBP1) (1.5) (Table 2).

Immunoblotting showed a dose-dependent decrease in follistatin (FS288 isoform) and an increase in BMP4 expression after rapamycin exposure supplementing the findings in Northern blot analysis (Fig. 4). Although Northern blotting did show follistatin expression in LNCaP cells and a reduction in mRNA under hypoxia and after rapamycin exposure, no follistatin (FS288) protein expression was detected by immunoblotting in LNCaP. LNCaP cells did not show a change in Smad4 in immunoblotting analysis after rapamycin exposure, hence, only data on PC3 cells are shown (Fig. 5). Smad-reporter assays showed a 4.5-fold increase in Smad activity in PC3 cells after exposure to rapamycin for 48 h (Fig. 6). In LNCaP cells Smad activation was significantly less than in PC3 cells, suggesting that the rapamycin-induced Smad activity in LNCaP cells plays a less prominent role in growth inhibition.

Table 1 Genes regulated by rapamycin at 24 h and hypoxia in both LNCaP and PC3. A value < 1 indicates down-regulation, values > 1 indicate overexpression at the mRNA level. All expression levels are relative to normoxic conditions without rapamycin

Gene	LNCa P			PC3			LNCaP hypoxia	PC3 hypoxia
	10	20			20	50		
Follistatin	0.98	0.89	0.88	0.89	0.61	0.61	0.87	0.71
eIF4E	0.75	0.65	0.71	0.63	0.55	0.34	0.81	0.43
FK506 binding protein 12 kDa (FKBP1/12)	1.07	1.12	1.21	1.68	1.71	1.81	0.65	1.57
Glucose-6-phosphate dehydrogenase	–	–	–	0.51	0.61	0.87	0.98	1.33
Glyceraldehyde 3-phosphate dehydrogenase	0.98	0.71	0.65	1.05	0.89	0.78	1.40	1.65
Lactate dehydrogenase-A	0.58	0.44	0.49	0.89	0.78	0.68	1.31	1.93
Mitochondrial ATP synthase subunit 9	0.71	0.66	0.74	0.61	0.71	0.81	0.55	1.14
G13 protein	0.84	0.84	0.77	0.85	1.25	0.95	0.72	0.93
ATP binding protein	0.81	1.19	0.93	0.5	0.51	1.12	1.07	0.35
Heat-shock 10 kDa protein 1 (chaperonin 10)	0.79	0.61	0.69	0.53	0.61	0.63	0.75	1.20
Human superoxide dismutase (SOD-1) mRNA, complete coding sequence	1.51	1.52	1.62	1.13	1.05	1.07	1.35	1.14
Carcinoembryonic antigen precursor	1.17	1.08	0.93	2.01	1.94	1.98	.65	1.56
Bone morphogenetic protein-4	–	–	–	1.23	1.55	1.72	–	0.61



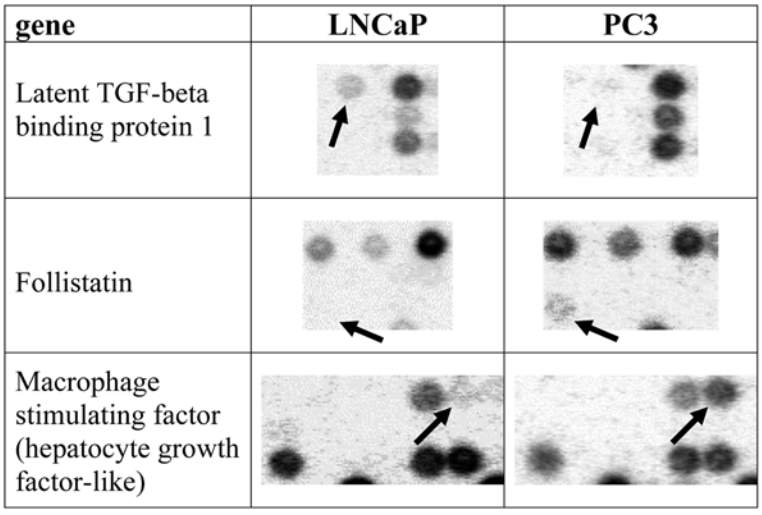


Fig. 2a–c Background and loading-corrected absolute expression levels (relative light units, RLU) of differentially expressed genes in LNCaP and PC3 cells (**a**). Relative expression levels (**b**). **c** Fragment of the GF211 filter array presenting the almost absent expression of follistatin mRNA in LNCaP as compared with PC3 cells

Table 2 Transforming growth factor (TGF)beta superfamily and related proteins expressed in LNCaP and PC3 cells. Ratio represents comparison of no versus 20 nM rapamycin and comparison of expression in baseline levels in LNCaP and PC3 cells (TGFbeta superfamily and related genes of which mRNA expression is not found to be influenced by rapamycin in LNCaP and PC3: TGFbeta receptorIII, latent TGF beta binding protein 2, bone morphogenetic protein (BMP)3b, TGFbeta2, BMP1, BMP6, BMP7, BMP8)

	LNCaP 0 vs 20 nM rapamycin	PC3 0 vs 20 nM rapamycin	LNCaP vs PC3
Follistatin	0.78	0.61	0.28
BMP2	1.11	0.61	1.65
TGFbeta receptor II	1.27	0.87	2.89
TGFbeta 3	–	0.9	
BMP receptor type II	1.11	0.98	1.81
Activin	1.05	1.17	0.97
Latent TGFbeta binding protein 1	0.89	1.51	0.28
BMP4	–	1.55	

Since rapamycin is involved in signal transduction through PI3K and AKT, and cross-talk exists between these pathways, we postulated that combined inhibition of signaling pathways may further enhance growth inhibition. To study the effects of inhibition of several signal transduction pathways on cell growth, rapamycin was combined with LY294002 (PI3K inhibitor), PD098059 (MAPK inhibitor) and FTI. Growth inhibition was determined using the MTT assay. Maximal inhibition was obtained by the combination of LY294002 and rapamycin in both LNCaP and PC3 (Fig. 7).

Discussion

Rapamycin is an immune suppressive agent with bone-preserving properties [22]. Studies showed the induction of the osteoblastic growth factor osteocalcin by

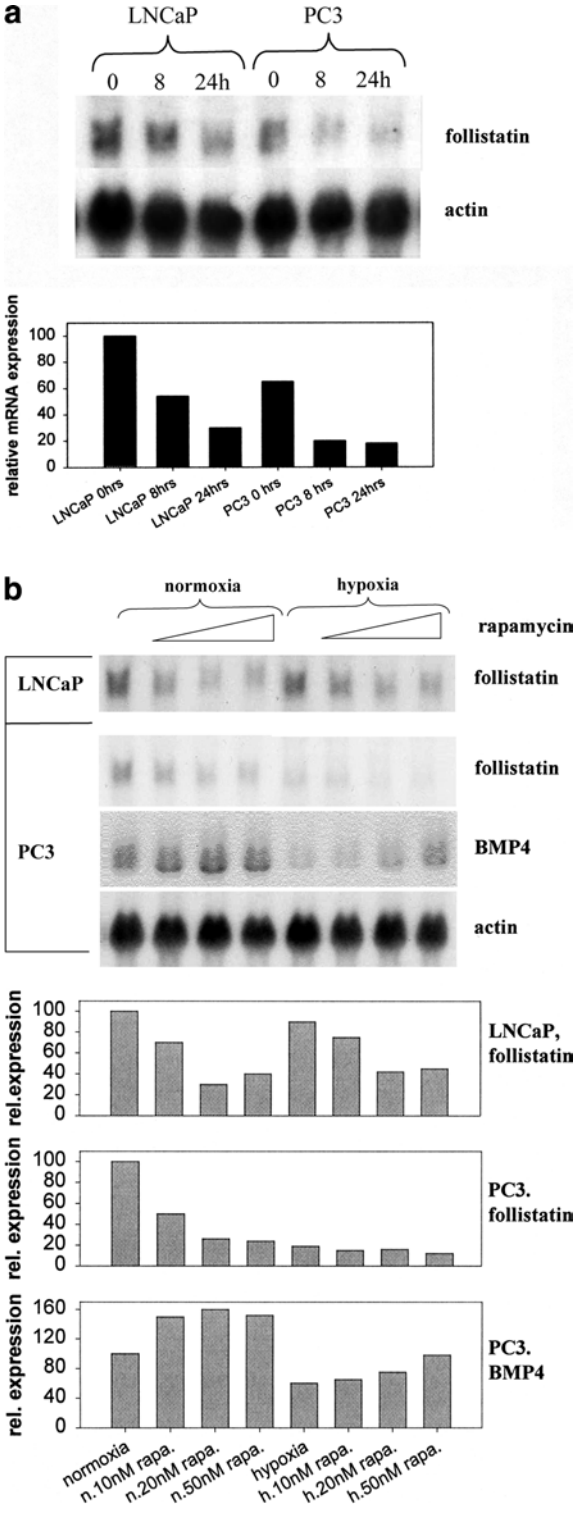


Fig. 3 **a** Follistatin mRNA expression decreased under hypoxia in both LNCaP and PC3 cells. **b** Follistatin and BMP4 mRNA expression in LNCaP and PC3 cells after 24 h rapamycin (0, 10, 20, 50 nM) exposure with and without hypoxia

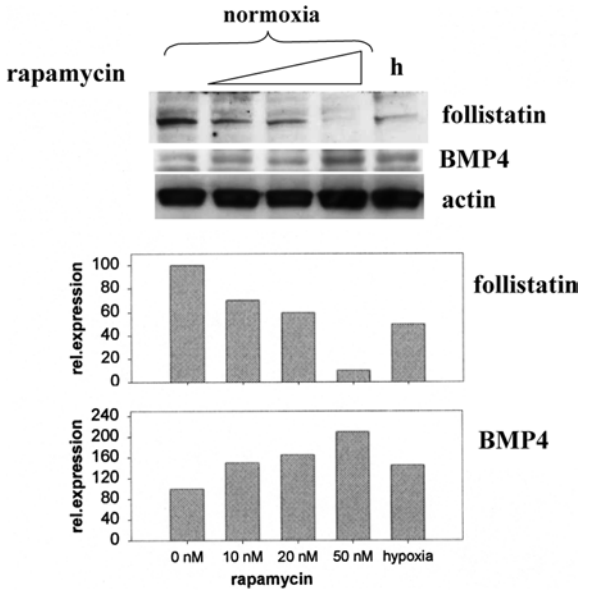


Fig. 4 Follistatin and BMP4 protein expression in cytoplasmic extracts from PC3 cells after 24 h rapamycin exposure (0, 10, 20, 50 nM) and hypoxia (*h*)

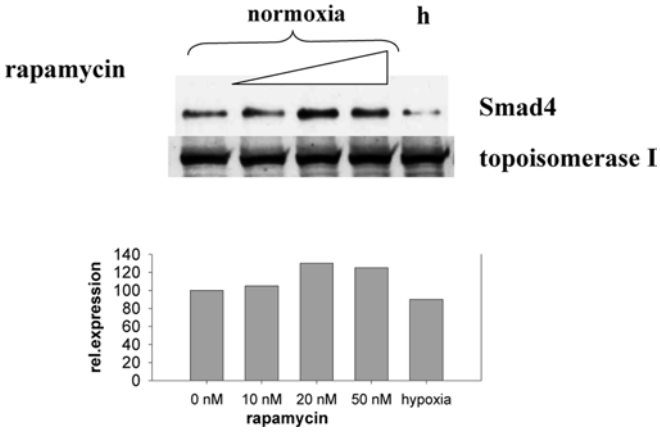


Fig. 5 Nuclear protein expression of Smad4 in nuclear extracts from PC3 cells. Different concentrations of rapamycin (0, 10, 20, 50 nM) and hypoxia (*h*)

rapamycin [19]. Growth inhibition by rapamycin was more pronounced in PC3 cells than in LNCaP cells. It is shown here that rapamycin also induces BMP4 expression and represses follistatin in PC3 cells. BMP4 is a member of the TGFbeta superfamily to which activin and TGFbeta 1 also belong. BMPs and their inhibitors such as noggin, chordin, and follistatin play an important role in embryological development [8, 11]. BMPs were overexpressed in particular in bone metastatic PCa in which BMP4 was found most dominantly expressed [3, 10] and, unlike BMP7, not androgen responsive [24]. For all members of the TGFbeta superfamily, downstream signaling after binding to the serine/threonine kinase receptors occurs through the Smad proteins. The final step in Smad signaling of the BMPs is the nuclear translocation of Smad4 [11]. Unlike pancreatic cancer, Smad4 is rarely mutated in PCa [16]. Moreover, Smad4 is induced by castration in both normal prostate and prostate cancer cells [4]. From the present data, it seems clear that rapamycin induces BMP4 and suppresses

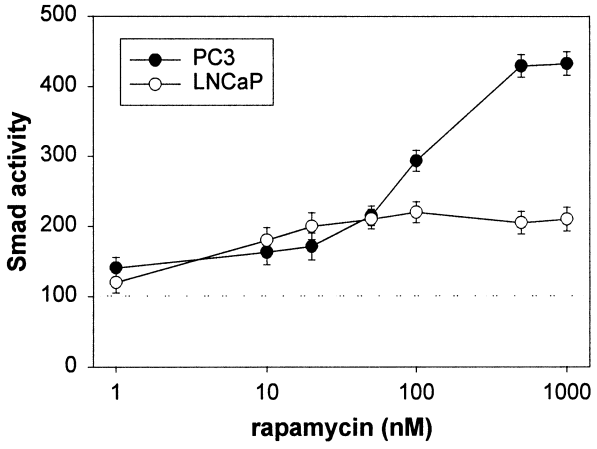


Fig. 6 Smad-reporter assay results. All data were normalized for transfection efficacy (CMV-beta-gal) and translatory inhibition (CMV-luciferase). *Error bars* present SE of mean for three different separate experiments for each cell line

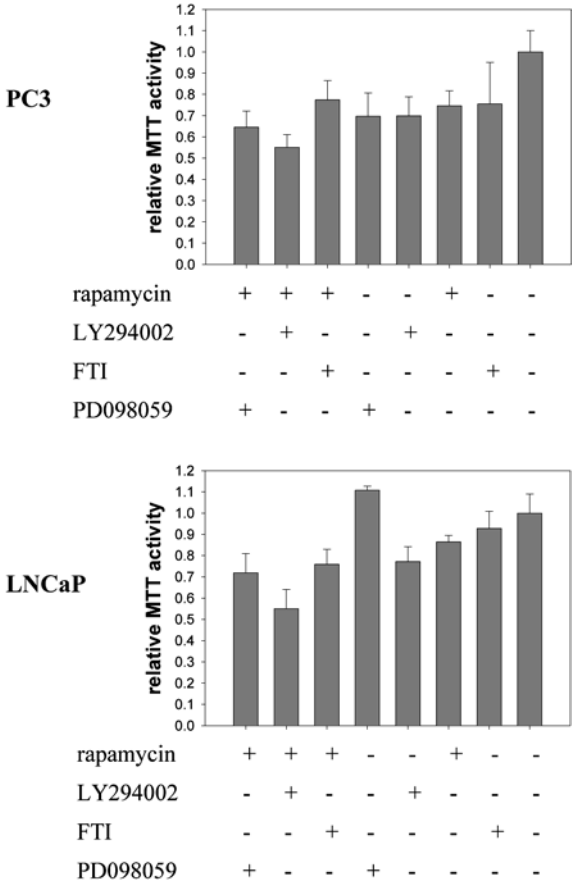


Fig. 7 Growth inhibition by several drugs and drug combinations assessed by MTT assay in RPMI medium containing 10% FBS. Values are normalized for no-drug (*last lane*). *RA* rapamycin (50 nM), PD98059 (100 μM), LY294002 (10 μM), *FTI* farnesyl transferase inhibitor(50 μM)

follistatin expression in the hormone-independent human prostate cancer cell line PC3, which resulted in an increase in Smad activity.

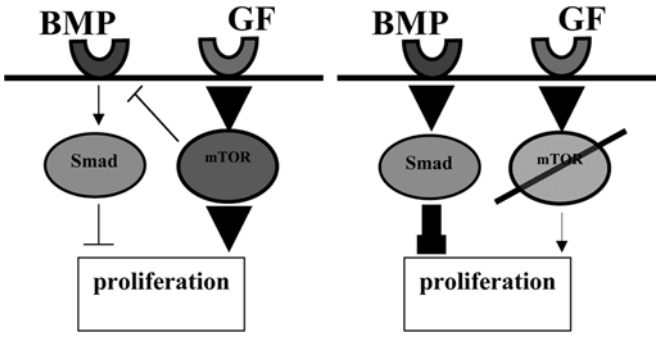


Fig. 8 Postulated mechanism of growth inhibition by rapamycin through mTOR inhibition. *BMP* bone morphogenetic protein, *GF* growth factor pathway

Besides its inhibitory effect on the FKBP12/mTOR complex, rapamycin was shown earlier to reverse the inhibitory effects of FKBP-12 on the TGFbeta type 1 receptor phosphorylation [27]. These findings suggest that the inhibitory effects of rapamycin may be exerted (partially) through TGFbeta (superfamily) signaling, either through BMP4 and/or TGFbeta receptor activation. Earlier findings showed an inhibitory effect of activin on prostate epithelium that could be neutralized by follistatin [18, 26]. Here we show a marginal increase in activin expression after exposure to rapamycin in both LNCaP and PC3 cells, whereas follistatin expression was upregulated. This may further activate Smad signaling and account for the growth inhibition of rapamycin in PCa. The lower Smad activation after rapamycin exposure in LNCaP cells compared with PC3 cells was associated with a reduced growth inhibition in the former cell line. Since it was recently shown that the androgen receptor (AR) interacts with and inhibits Smad3, the role of AR in the varying response to rapamycin between LNCaP and PC3 cells should be a topic of further study [5].

PTEN expression was recently shown to be downregulated by TGFbeta 1, indicating cross-talk between the TGF-Smad and AKT pathways [7]. To evaluate if PI3K- and MAPK inhibition would influence the growth inhibitory effects of rapamycin, we combined PI3K inhibition (LY294002), MAPK inhibition (PD098059), and FTI. The strongest additional inhibition was seen for the combination of PI3K inhibition (LY294002) and rapamycin. The fact that PI3K/AKT activation was shown earlier to inhibit Smad-regulated caspase expression may explain the additional growth inhibitory effect of LY294002.

To our knowledge, this is the first time that mTOR inhibition using rapamycin was shown to induce BMP4 expression, to decrease follistatin expression, and to activate Smad signaling (Fig. 8). Induction was sustained after 24 h of exposure to rapamycin at different concentrations but was markedly decreased under hypoxic conditions, as was the overall BMP4 expression. Combining LY294002 (PI3K inhibitor) and rapamycin enhanced the growth inhibition in PCa cell lines. These findings show that part of the growth inhibitory effects

of rapamycin may be exerted through BMP/activin/Smad activation. In particular, the improved cytotoxicity of a combination of PI3K and mTOR inhibition deserves further evaluation as potential anticancer agents in prostate cancer.

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