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FAST TRACK

Cyr61 downmodulation potentiates the anticancer effects of zoledronic acid in androgen-independent prostate cancer cells

Monica Marra[1], Daniele Santini[2], Giuseppina Meo[1], Bruno Vincenzi[2], Silvia Zappavigna[1], Alfonso Baldi[1], Maciej Rosolowski[3], Giuseppe Tonini[2], Markus Loeffler[3], Ruth Lupu[4], Santolo Rosario Addeo[1], Alberto Abbruzzese[1], Alfredo Budillon[5] and Michele Caraglia[1,5]*

[1]*Department of Biochemistry and Biophysics, Second University of Naples, Naples, Italy*
[2]*Department of Medical Oncology, University Campus Bio-Medico, Rome, Italy*
[3]*Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany*
[4]*Department of Medicine, Evanston Northwestern Research Institute, Feinberg Medical School, Evanston, IL*
[5]*Experimental Pharmacology Unit, National Cancer Institute of Naples ''Fondazione G. Pascale'', Naples, Italy*

We have analyzed the gene modulation induced by zoledronic acid (ZOL) in androgen-resistant prostate cancer PC3 cells with cDNA microarray platform to identify new molecular targets of ZOL in prostate cancer. The gene coding for cysteine-rich, angiogenic inducer, 61 (CYR61) resulted highly downregulated with a fold change of 5.58. Therefore, we have studied the effects of ZOL on CYR61 protein product, and we have found that CYR61 protein expression was decreased significantly after exposure to ZOL. The effect of ZOL on CYR61 expression was dose and time dependent was due to a reduced transcriptional activity of CYR61 promoter. Moreover, the effects induced by ZOL were paralleled by decreased activation of Ras-Raf-1- and Akt-dependent pathways that was dependent from isoprenylation inhibition, since it was antagonized by the addition of geranylgeraniol. Finally, we have investigated the role of CYR61 in the regulation of growth inhibition and invasion/motility of PC3 cells using a shRNA for CYR61 to downregulate the expression of CYR61 protein. The enhanced inhibition of proliferation and motility/invasion induced by ZOL by S-phase accumulation. In the same experimental conditions, CYR61 protein downregulation potentiated the inactivation of the Ras-dependent proliferation pathway and cell cycle inhibitors p21 and p27 expression.

Key words: CYR61; zoledronic acid; isoprenylation; prostate cancer

The cysteine-rich protein 61 (CYR61/CCN1) belongs to the family of CYR61/CTGF/Nov (CCN) proteins, secreted matricellular proteins whose expression is rapidly and transiently induced in response to growth and stress stimuli.[1–3] CYR61/CCN1 is a 40-kDa cysteine-rich and heparin-binding protein that either localizes intracellularly or associates with extracellular matrix and cell surfaces. Functionally, CYR61 promotes cell adhesion, proliferation, migration and angiogenesis through cell type-specific binding to different integrins like $\alpha 6\beta 1$ and $\alpha v\beta 3$.[4,5] The CYR61 protein activities are mediated potentially through interactions with membrane proteins such as heparan sulfate proteoglycans, other growth factor receptors, integrins and/or through other incompletely characterized nonintegrin receptors.[5,6] Changes in CYR61 expression have been associated with several cancer types.[7,8] CYR61 was also reported to induce migration and invasion of tumor cells,[9] and a role for CYR61 has been proposed in wound healing and tumor development, two events in which hypoxia is a vital component.[10] However, the mechanisms of CYR61 transcriptional regulation are less well understood.

Zoledronic acid (ZOL) is an aminobisphosphonate able to inhibit the prenylation of intracellular proteins through the inhibition of farnesylpirophosphate synthase. ZOL is indicated for the treatment of skeletal complications secondary to bone metastases derived from solid tumors including hormone-refractory prostate cancer, breast cancer, lung cancer and renal cell carcinoma.[11,12]

Several *in vitro* studies have shown that ZOL inhibits adhesion of tumor cells to extracellular matrix (ECM) proteins, thereby impairing the process of tumor-cell invasion and metastasis.[13,14] Recent data indicate that inhibition of tumor-cell adhesion to ECM proteins is dependent on inhibition of protein prenylation. Therefore, inhibition of the mevalonate pathway and induction of caspase activity are important for the inhibitory effects of ZOL. Furthermore, it has been shown that an activating Ras mutation enhanced the adhesion of a normal breast epithelial cell line to ECM proteins, suggesting that increased Ras activation may increase the metastatic potential of breast cancer cells. Thus, by inhibiting protein prenylation and Ras signaling, ZOL should reduce the ability of tumor cells to expand once they colonize bone.[14–17]

Evidence from *in vitro* and *in vivo* models indicates that ZOL synergizes with a variety of anticancer agents including chemotherapeutic drugs, molecular targeted agents and other biological agents.[17–26]

The available preclinical data on nitrogen-containing bisphosphonates, as ZOL do not provide final conclusions about both their biological/biochemical activity and on precise intracellular molecular targets. The identification of genes modulated by ZOL could be useful to design new therapeutic strategies and to potentiate the antitumor effects of these substances. In this view, gene profiling in preclinical experimental models (*in vitro* tumor-cell lines maintained in standardized conditions) could be useful to elucidate the mechanisms of action of anticancer drugs (*e.g.*, ZOL) and to define the molecular targets of this agent understanding the biological consequences of these molecular changes.

In this report, we used the DNA microarray technique to analyze the gene expression pattern of androgen-independent prostate cancer PC3 cells exposed to ZOL to identify possible molecular targets responsible for ZOL antitumor activity. CYR61 was demonstrated downregulated in this experimental model. We have evaluated the effects of CYR61 knock down through the use of shCYR61 plasmids on CYR61 expression, proliferation, apoptosis, motility and invasiveness in combination or not with ZOL in PC3 cells.

Material and methods

Materials

RPMI, BSA and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Microtech

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***Correspondence to:** Department of Biochemistry and Biophysics, Second University of Naples, Via Costantinopoli 16, Naples 80138, Italy. Fax: +00390815665863. E-mail: michele.caraglia@unina2.it

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(Naples, Italy). Rabbit antisera raised against Erk1/2 and CYR61, monoclonal antibodies (mAb) raised against p21 and GAPDH and phycoerythrin-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antisera raised against Akt, pAkt and mAb raised against phospho-Erk1/2 were purchased by Cell Signalling (Cell Signaling Technology, MA). Anti-pan-Ras clone 10 MAb, and the relative Ras activation assay kit was purchased from Calbiochem (Darmstadt, Germany). Matrigel™ was purchased from Sigma AG (Sigma-Aldrich srl, Milano, Italy). ZOL was a gift of Novartis (Novartis, Basel, Switzerland). R115777 was a gift of Orthobiotech (Orthobiotech, Janssen Research Center, Raritan, NJ). BAY-439006 was purchased from Bayer Co. (Milan, Italy) and gefitinib from AstraZeneca (Milan, Italy).

Cell cultures

The human prostate androgen-independent PC3 and DU145 and androgen-dependent LNCaP cell lines obtained from the American Type Tissue Culture Collection, Rockville, MD, were grown in DMEM supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Gene array

Gene signatures of untreated *versus* ZOL-treated cells were obtained by Affymetrix HG-U133 chips (including more than 33,000 well-known human genes) according to the manufacturer's recommendations, as previously described.[27] Probe intensities were normalized using a variance stabilization method.

Preprocessing methods were performed in 2 independent examinations. The subsequent analysis included methods as analysis of variance (ANOVA), Local Pooled Error which is specially designed for small-sample experiments and Local False Discovery Rate. All the quality controls performed showed no problems. We considered only the genes with fold changes >3 with the statistical difference of expression of at least $p < 0.001$.

Western blotting

Cells were grown for different times with or without increasing concentrations of ZOL at 37°C. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped and centrifuged for 30 min at 4°C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 M NaCl, 1mM EDTA, pH 7.5, 10 mM Na_2HPO_4, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 units/ml aprotinin). Equal amounts of cell proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose and reacted with the different antibodies. Blots were then developed using enhanced chemoluminescence detection reagents (SuperSignal West Pico, Pierce) and exposed to X-ray film. All films were scanned by using Quantity One software (BioRad laboratories, Hercules, CA).

Affinity precipitation of Ras

After treatement with ZOL and/or farnesol (FOH) or geranylgeraniol PC3 cells were lysed in the 1× Mg^{2+} buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA and 2% glycerol). Then, 10 μl Ras Binding Domain conjugated to agarose was added to 1 mg of cell lysate, and the mixture was incubated at 4°C for 1 hr. The agarose beads were collected by microcentrifugation at 14,000*g* for 5 sec and washed 3 times with Mg^{+2} buffer. The detection of the expression of active Ras was performed as previously described.[28]

Membrane surface detection of CYR61

For determination of cell surface expression of CYR61, fluorescence-activated cell sorting (FACS) analysis was performed using indirect staining of CYR61. After washing with cold PBS 1×, cell pellets were incubated with anti-CYR61 mAb (10 μg/sample) for 30 min at 4°C in the dark. The cells again were washed with PBS and incubated with Phycoerythrin-conjugated antimouse IgG (25μl/sample) for 30 min at 4°C in the dark. After washing, FACS sorting was performed using a FACScan (Becton Dickinson, Mountain View, CA), and analysis was performed using CellQuest 2.0 (Becton Dickinson).

Luciferase assay

The CYR61 promoter activity in PC3 untreated and ZOL-treated cells was determined by luciferase assay. In details, cells were transfected transiently using Lipofectamine 2000 reagent in according to the manufacturer's instructions (Invitrogen) with the CYR61-pGL3 full length promoter plasmid or with the empty vector pGL3 kindly provided by Ruth Lupu of Evanston Northwestern Research Institute (IL). After 48 hr from treatment with ZOL, luciferase activity was measured using Dual Light® System (Biosystems, Milan, Italy) according to the manufacturer's instructions. Luminescent signal was quantified by the Tecan's Infinite™ M200 multimode reader (Tecan Italia, Milan, Italy) equipped with a 2 channel injector.

Transfection of PC3 cells with CYR61 short hairpin RNA

To establish the specific role of CYR61, PC3 cells were transfected with 4 predesigned CYR61 short hairpin RNA (shRNA) vectors or scrambled vector (SureSilencing, SuperArray, Frederick, MD), according to the manufacturer's instructions. To isolate neomycin-resistant colonies, 5 μg/mL Geneticin (Invitrogen) selection was applied. Silencing of CYR61 expression was confirmed by reverse transcription-PCR analysis and immunoblot.

Cell proliferation assay

Proliferation of PC3 parental and transfected cells was performed in the presence of ZOL by MTT assay. Briefly, cells (3×10^4) were seeded in 96-well plates in serum-containing media and allowed to attach for 24 hr. The medium was then removed and replaced with new medium containing drugs at different concentrations. Cells were incubated under these conditions for a time course spanning 72 hr. Then cell viability was assessed with MTT method as previously described.[28]

Cell cycle analysis

PC3 parental and transfected cells were seeded in 100 mm plates at the density of 1×10^6 cells/plate. After incubation with ZOL cells were washed in PBS, pelleted and directly stained in a propidium iodide (PI) solution (50 μg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 30 min at 4°C in the dark. Flow cytometry analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To evaluate cell cycle PI fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 20,000 events for each point were analyzed in at least 3 different experiments giving a S.D. less than 5%.

RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Total RNA of 1 μg was DNase-I- (Invitrogen, Milan, Italy) treated, and cDNA was synthesized using SuperScript® III RT (Invitrogen). This cDNA of 2 μl was subsequently used for analysis by PCR. The following primer set was used: CYR61 sense 5′-AGGTGGAGTTGACGAGAAAC-3′ and antisense 5′-ACTGGATCATCATGACGTTCT-3′. PCR reaction of 25 μl was subjected to 25 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C followed by a 10 min extension at 72°C. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Band intensities of target genes relative to GAPDH control were calculated using NIH Image J.

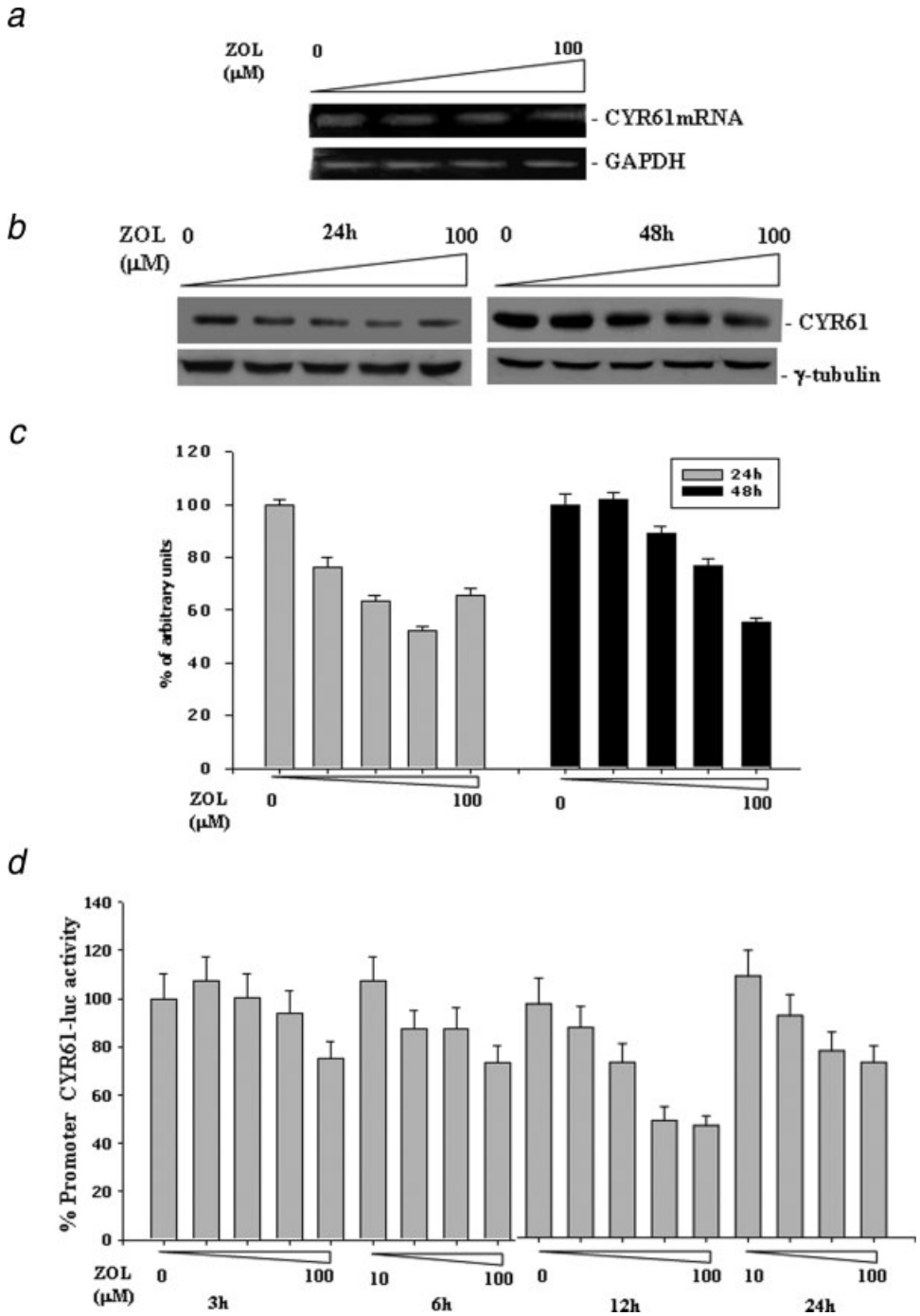


FIGURE 1 – Regulation of CYR61 mRNA and protein by ZOL on PC3 cell line. *a*) RT-PCR for CYR61 was performed as described in ''Material and Methods''. CYR61 mRNA was downregulated in a dose-dependent manner after 24 hr of ZOL treatment with 0, 25, 50, 100 μM. Expression of the house-keeping gene GAPDH was used as loading control. *b*) Determination of the expression of CYR61 was evaluated after blotting with an anti-CYR61 specific Mab as described in ''Material and Methods''. PC3 cells were exposed to increasing concentrations of ZOL (0, 10, 25, 50, 100 μM) for 24 and 48 hr. CYR61 protein expression was decreased significantly in a time and dose-dependent manner. Expression of the house-keeping protein γ-tubulin was used as loading control. *c*) Laser scanner of the bands associated to CYR61 protein expression. The intensities of the bands, normalized against loading control, were expressed as % of arbitrary units. Bars, SEs. The experiments were performed at least 3 different times and the results were always similar. *d*) Luciferase assay for transcriptional activity of CYR61 promoter was performed as described in ''Material and Methods''. PC3 cells were transfected with pGL3-CYR61-LUC and treated with increasing concentrations of ZOL (0, 10, 25, 50, 100 μM) at different times (3, 6, 12, 24 hr). Bars, SDs. The experiments were performed 3 different times, and the results are the mean of data obtained in the different experiments.

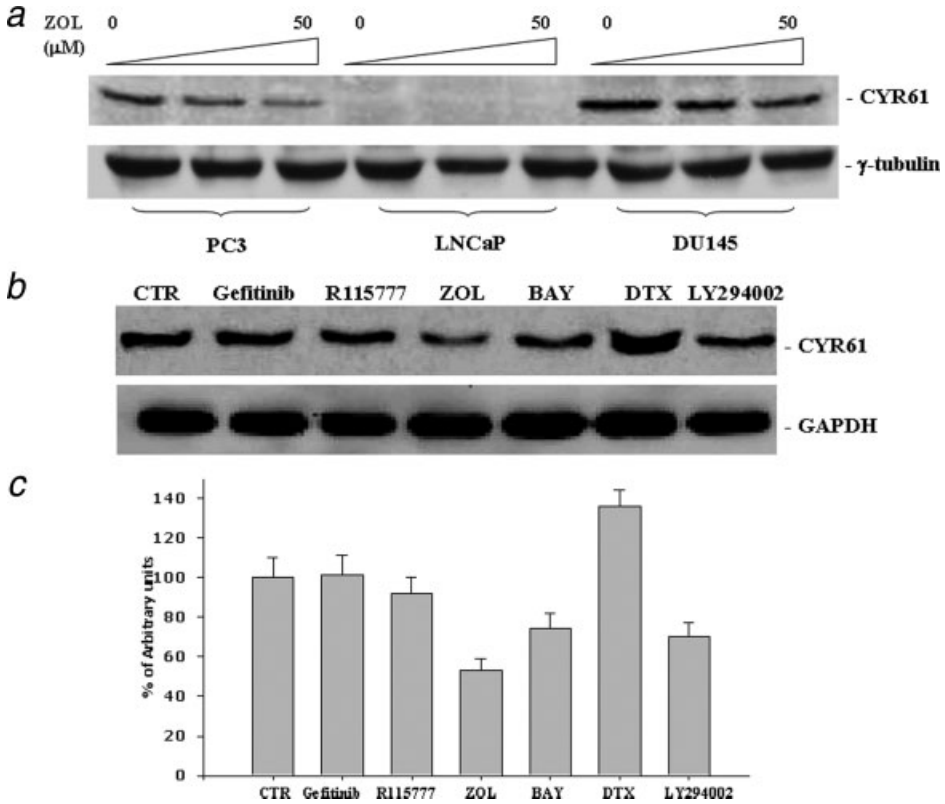


FIGURE 2 – Effect of ZOL on CYR61 protein expression in human prostate cancer cell lines *a*) Effect of ZOL on CYR61 protein expression in human androgen-independent PC3 and DU145 and androgen-dependent LnCaP prostate cancer cells. Determination of the expression of CYR61 was evaluated after blotting with an anti-CYR61 specific antibody as described in ''Material and Methods''. CYR61 protein expression was downregulated in a dose-dependent manner after 48 hr of ZOL treatment at 25 and 50 μM concentration in both PC3 and DU145 cells, whereas androgen-dependent LNCaP cell line did not express detectable levels of CYR61. The house-keeping protein γ-tubulin was used as loading control. The experiments were performed at least 3 different times, and the results were always similar. *b*) Effects of other specific inhibitors of the Ras→Raf-dependent pathway used at their own 50% growth inhibitory concentration at 48 hr on PC3 cell line. ZOL and inhibitors of the EGF-R-associated tyr kinase (gefitinib) or of farnesyltransferase (R115777) or of Raf-1 (BAY 43-9006) or of PI3K (LY294002) induce less effects on CYR61 modulation if compared with ZOL. The house-keeping protein GAPDH was used as loading control. The experiments were performed at least 3 different times, and the results were always similar. *c*) Laser scanner of the bands associated to CYR61 protein expression of PC3 cells treated with the different inhibitors. The intensities of the bands were expressed as % arbitrary units. Bars, SEs. The experiments were performed at least 3 different times, and the results were always similar. CTR: untreated PC 3 cells; gefitinib: PC cells treated with 200 μM gefitinib for 48 hr; R115777: PC3 cells treated with 11 μM R115777 for 48 hr; ZOL: PC3 cells treated with 15 μM ZOL for 48 hr; BAY: PC3 cells treated with 8 μM BAY 43-9006 for 48 hr; DTX (docetaxel): PC3 cells treated with 10 ng/ml DTX for 48 hr; LY294002: PC3 cells treated with 10 μM LY294002 for 48 hr.

Invasion and motility assays

For motility assays, 5×10^5 cells were plated in the top chamber of noncoated polyethylene teraphthalate (PET) membranes (6-well insert, pore size 8 μm; Becton Dickinson). For *in vitro* invasion assays, Matrigel was diluted to 1 mg/ml in serum-free RPMI medium. Matrigel (100 μl of 1 mg/ml) were placed on the lower side of each insert (24-well insert, pore size 8 μm; Becton Dickinson). The insert and the plate were incubated overnight at 4°C. The following day, cells were harvested and suspended in RPMI at a concentration of 1×10^6 cells/ml. The inserts were washed with serum-free RPMI, then 1×10^6 cells was added to each insert, and 3 mL of RPMI containing 10% FCS were added to the well underneath the insert. Cells were incubated at 37°C up to 24 hr. After this time, the inner side of the insert was wiped with a wet swab to remove the cells, whereas the outer side of the insert was gently rinsed with PBS and stained with 0.25% crystal violet for 10 min, rinsed again and then allowed to dry. The inserts were then viewed under a CCD camera equipped Nikon microscope. The inserts were also processed, and the cells labeling was detected by a spectrophotometer: the crystal violet was extracted with 900 μl of 0.1 M sodium citrate in 50% ethanol. The absorbance was measured at 585 nm.

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed by ANOVA with Neumann-Keul's multiple comparison test or Kolmogorov-Smirnov where appropriate.

Results

Microarray analysis of ZOL-modulated genes identifies CYR61 as a downregulated gene

We have evaluated the expression profiling of androgen-independent prostate cancer PC3 cells treated with 100 μM ZOL for 24 hr *versus* untreated using Affymetrix HG-U133 chips (as described in ''Materials and Methods'') to identify possible molecular targets of ZOL antitumor action.

The upregulated and downregulated genes were 73/33.000 (we considered only the genes with fold changes >3), among these, 4 genes were downregulated (−3.82 to −5.58), and 69 genes were upregulated (+3 a +45.52). The statistical difference of expression of each gene was at least $p < 0.001$. Among the downregulated genes, the gene coding for CYR61 resulted downregulated highly with a fold change of 5.58. CYR61 overexpression in tumor cells promotes tumor growth and vascularization. These results

suggest that inactivation of CYR61 by novel approaches may provide optimal therapeutic strategies for the treatment of prostate cancer, and its reduction induced by ZOL requires further investigation.

CYR61 is downregulated in a time- and dose-dependent manner both at mRNA and protein levels

To further substantiate the alterations of gene expression obtained by microarray analyses, time-course experiments were performed for CYR61 expression using both RT-PCR and Western blot. The results of RT-PCR for CYR61 was in direct agreement with the microarray data (Fig. 1*a*), though the fold changes in the expression level was not exactly the same between these two different analytic methods. The results of Western blot analysis were also in agreement with the microarray and RT-PCR data. In fact, we have found that CYR61 protein expression was decreased significantly after both 24 hr (50%) and 48 hr (45%) exposure to 50 μM ZOL (Fig. 1*b*). On the basis of these data, we have selected this concentration of ZOL for all the subsequent experiments even if the effect was more pronounced with greater concentrations of ZOL (Fig. 1*b*). These results support the findings obtained from microarray experiments, supporting and providing molecular evidence for the involvement of CYR61 in angiogenesis and differentiation of human prostate cancer cells.

Transcriptional activity of CYR61 promoter in PC3 cells treated with ZOL

To assess the effects of increasing concentrations of ZOL for 3–24 hr on CYR61 transcriptional activity, we transfected PC3 cells with a construct (pGL3-CYR61-LUC) in which the luciferase reporter gene is driven by the CYR61 promoter. PC3 cells were treated with increasing concentrations of ZOL at different times and assessed for luciferase activity. ZOL (50 μM) suppressed significantly luciferase activity (about 50%) driven by the CYR61 promoter in the pGL3-CYR61-LUC construct with a peak at 12 hr from the beginning of the exposure (Fig. 1*c*).

CYR61 expression is modulated by ZOL also in hormone-independent prostate cancer DU145 cells

To assess if the effects of ZOL occurred also on other androgen-independent prostate cancer cells, we have studied the effects of different concentrations of ZOL on CYR61 protein expression in human androgen-dependent LnCaP and androgen-independent PC3 and DU145 prostate cancer cells. CYR61 protein expression was about 1.5- or 2-fold decreased after exposure of both PC3 and DU145 cells to ZOL for 48 hr, whereas androgen-dependent LNCaP cell line did not express detectable levels of CYR61. The downregulation of CYR61 protein expression was dose dependent, as evaluated by western blotting. The house-keeping protein γ-tubulin was used as loading control (Fig. 2*a*).

CYR61 downregulation is specifically induced by ZOL

We have investigated if the effects on CYR61 expression induced by ZOL were specific or if alternatively these effects were also caused by other specific inhibitors of the Ras→Raf-dependent pathway used at equitoxic concentrations (when 50% growth inhibition was recorded). Inhibitors of ras farnesylation (such as R115777) or of EGF-R-associated tyr kinase (gefitinib) or of C-Raf (BAY 43-9006) or of PI3K inhibitor (LY294002) used at equitoxic concentrations do not induce or induce less effects on CYR61 modulation if compared with ZOL. The most active agent appeared to be BAY 43-9006 that, however, induced only a 40% decrease of CYR61 expression. On the other hand, the cytotoxic drug docetaxel (DTX), which acts through the inhibition of the mitotic apparatus, caused an about 40% increase of CYR61 expression. The downregulation of CYR61 did not appear to be dependent, completely or in part, from either EGF-R or C-Raf or PI3K inhibition and by an unspecific growth inhibition due to exposure to cytotoxic drugs such as DTX (Fig. 2*b*). Also the partial inhibition of ras isoprenylation due to FTI R115777 is not able to significantly reduce CYR61 expression.

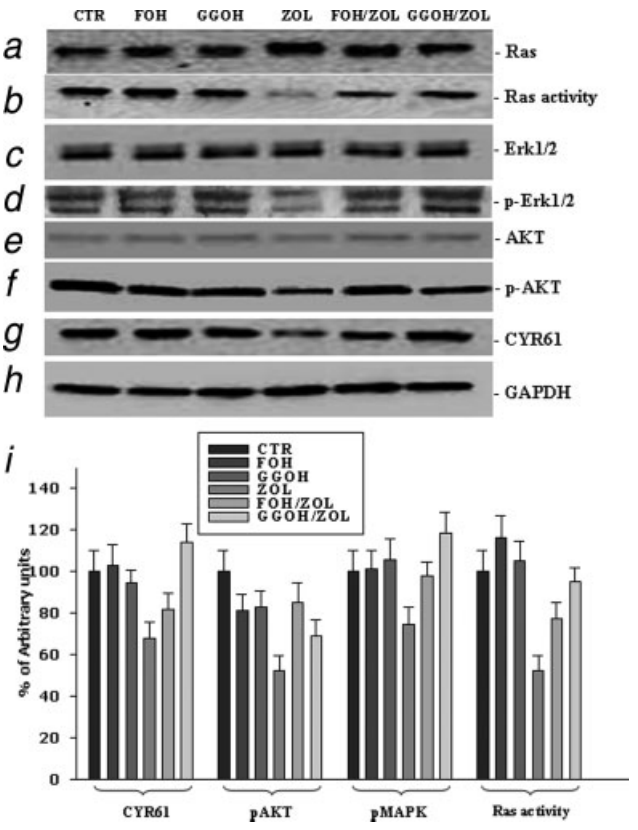
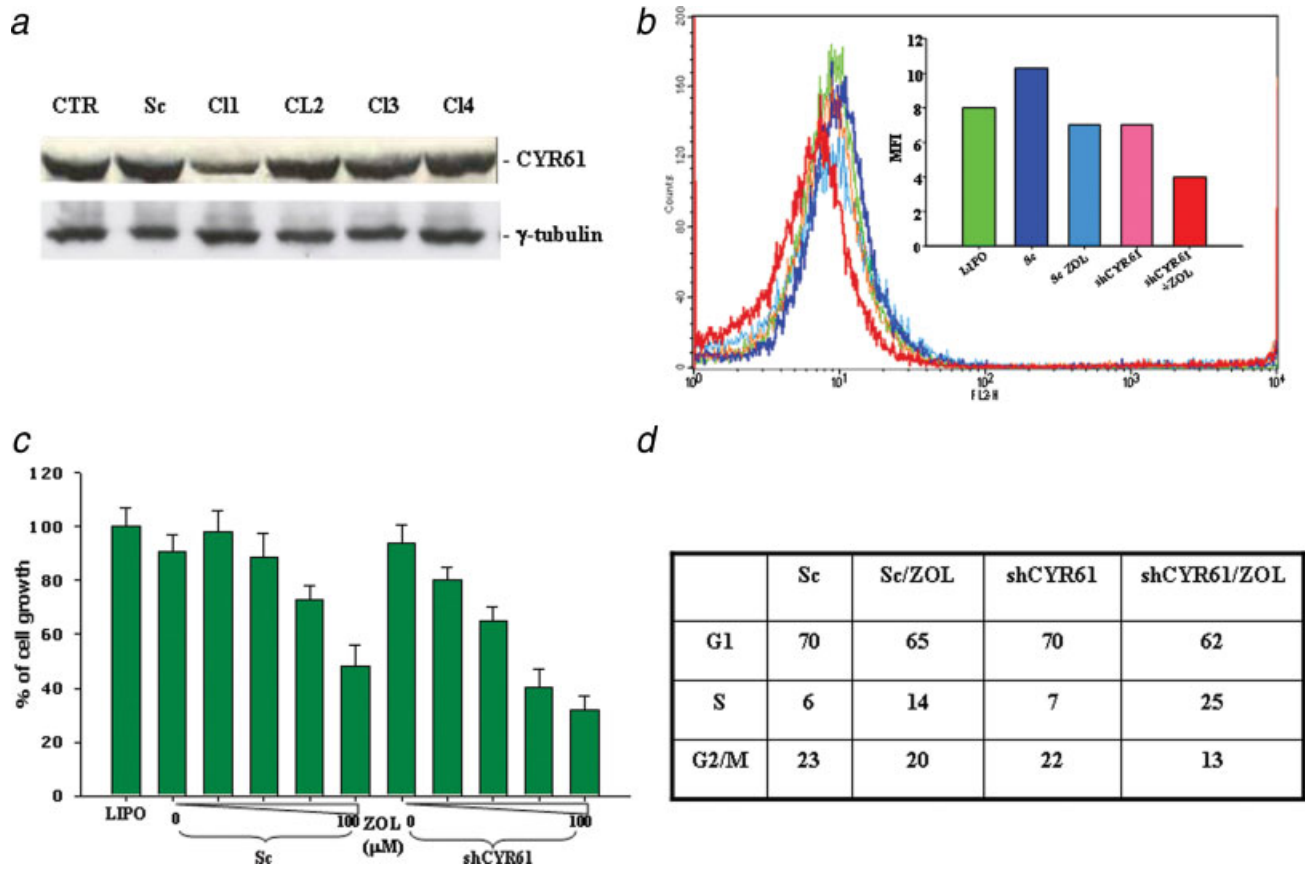


FIGURE 3 – Isoprenylation inhibition is involved in the regulation of CYR61 expression. PC3 cell line was treated with 50 μM ZOL for 48 hr in the presence or absence of either 10 μM farnesol or 10 μM geranylgeraniol. Thereafter, both the expression and activity of the components of the Ras→Erk and Akt-dependent pathways were evaluated. *a*) Western blot assay for the expression of the total Ras protein. *b*) Affinity precipitation of Ras performed with the minimal binding domain of raf-1 conjugated with agarose microspheres for the evaluation of Ras activity as described in ''Material and Methods''. Determination of the expression *c*) and phosphorylation *d*) of Erk-1/2 evaluated after blotting with specific antibodies, as described in ''Material and Methods''. Evaluation of the expression *e*) and activity *f*) of Akt analyzed after blotting with an anti-Akt and an anti-pAkt specific antibodies, respectively, as described in ''Material and Methods''. *g*) Expression of CYR61 evaluated after blotting with a specific antibody. *h*) Expression of the house-keeping protein γ-tubulin, used as loading control. *i*) Laser scanner of the bands associated to CYR61 expression and Ras, Erk and Akt activity. The intensities of the bands were expressed as % arbitrary units. Bars, SEs. The experiments were performed at least 3 different times, and the results were always similar. CTR: untreated PC 3 cells; FOH: PC3 cells treated with 10 μM FOH for 48 hr; GGOH: PC3 cells treated with 10 μM GGOH for 48 hr; ZOL: PC3 cells treated with 50 μM ZOL for 48 hr; FOH/ZOL: PC3 cells treated with 10 μM FOH and 50 μM ZOL for 48 hr; GGOH/ZOL: PC3 cells treated with 10 μM GGOH and 50 μM ZOL for 48 hr.

ZOL effects are dependent from geranyl-geranylation inhibition

We have investigated if the downregulation of CYR61 protein product was dependent from the action of ZOL on the mevalonate pathway and consequently on the ras-prenylation pathways. ZOL inhibited both Ras and Erk-1/2 activity without affecting their expression, as also previously reported[20] (Figs. 3*a*–3*d*). Similarly, ZOL did not induce any change of Akt expression although caused a reduction of its activity (Figs. 3*e* and 3*f*). In these experimental conditions, ZOL downregulated CYR61 expression (Fig. 3*g*). We have also added either 10 μM farnesol (FOH) or 10 μM geranyl-



	Sc	Sc/ZOL	shCYR61	shCYR61/ZOL
G1	70	65	70	62
S	6	14	7	25
G2/M	23	20	22	13

Figure 4 – Effect of CYR61 downregulation on the sensitivity of PC3 cells to antiproliferative effects induced by ZOL. *a*) PC3 cells were transfected with different clones of shCYR61. Clone 1 was able to induce the best downregulation of CYR61 protein expression after 24 hr of culture as shown by western blotting with anti-CYR61 antibody performed as described in ''Material and Methods''. This clone was selected for all the subsequent experiments. CTR: parental PC3 cells; Sc: PC3 cells transfected with scrambled vector; Cl1-4: PC3 cells transfected with the 4 shCYR61 clones. *b*) CYR61 surface expression on PC3 transfected cells evaluated with FACS analysis after labeling with a specific anti-CYR61 antibody as described in ''Material and Methods''. FACS analysis showed a decrease of CYR61 surface expression after treatment with 50 μM ZOL for 24 hr. The effects of ZOL on CYR61 expression of parental (Sc) PC3 cells were mild. The experiments were performed at least 3 different times, and the results were always similar. LIPO: PC3 cells exposed to lipofectamine for 24 hr; Sc: PC3 cells transfected with a scrambled vector and cultured for 24 hr without ZOL; Sc ZOL: PC3 cells transfected with scrambled vector and exposed for 24 hr to 50 μM ZOL; shCYR: PC3 cells transfected with shCYR61 and cultured for 24 hr; shCYR/ZOL: PC3 cells transfected with shCYR61 and exposed to 50 μM ZOL for 24 hr. *c*) PC3 parental and transfected cell were treated with increasing concentrations of ZOL (0, 10, 25, 50, 100 μM) for 24 hr. Cell proliferation was evaluated with MTT assay as described in ''Material and Methods''. Each point is the mean of 3 different evaluations performed in at least 3 different experiments. Bars, SDs. CTR: parental PC3 cells; LIPO: parental PC3 cells exposed to lipofectamine for 24 hr. *d)* Evaluation of the distribution of PC3 cells exposed to 50 μM ZOL for 24 hr in the different phases of the cell cycle. This experiment was performed at FACS analysis after labeling with PI as described in ''Material and Methods''. The treatment of PC3 cells transfected with shCYR61 with ZOL potentiated the increase of cells in S-phase (25%) and induced a decrease of cell accumulation in G_2/M phase (13%). Each point is the mean of 3 different evaluations performed in at least 3 different experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

geranyol (GGOH) to ZOL-treated and untreated PC3 cells to restore the intracellular isoprenoid levels that were decreased by the farnesyl pyrophosphate synthase inhibition induced by ZOL. We have found that the addition of either GGOH or FOH alone to PC3 cells did not change both the expression and activity of the signal transduction components and of CYR61. On the other hand, GGOH antagonized completely the effects of ZOL on both signal transduction and CYR61, whereas FOH caused a partial restoration of the biochemical modulation induced by ZOL. In these experimental conditions, FOH and GGOH had also counteracting effects on cell growth inhibition induced by ZOL. In fact, at 48 hr 50 μM ZOL caused an about 56% growth inhibition, whereas the addition of FOH reduced growth inhibition at 25% and GGOH antagonized completely cell growth inhibition induced by ZOL (data not shown). These results suggested strongly that the biological and biochemical effects induced by ZOL on prostate cancer PC3 cells were largely due to the inhibition of geranyl-geranylation more than farnesylation.

Effect of shCYR61 and ZOL on PC3 cell proliferation and cell cycle

CYR61 is a protein with pleiotropic functions involved in the regulation of transformed phenotype of eukaryotic cells. On the basis of these considerations, we have investigated the role of CYR61 in the anticancer effects induced by ZOL in human prostate cancer cells. We transfected transiently the PC3 cells with shRNA for CYR61 to downregulate the expression of CYR61 protein. As shown in Figure 4*a*, the clone 1 was the most active in inhibiting CYR61 protein expression giving an about 2.5-fold reduction of its expression. Moreover, the downregulation of the protein was maximal after 24–48 hr from the transfection and thereafter resembled that one of parental cells (data not shown). On the basis of these considerations, we choose the clone 1 to perform all the experiments after 24 or 48 hr of treatment with ZOL. CYR61 expression on transfected PC3 cells surface was decreased (1.5-fold) after treatment with 50 μM of ZOL for 24 hr, as

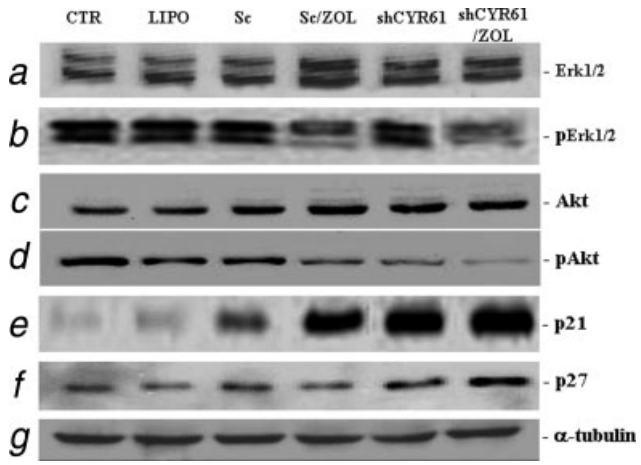


FIGURE 5 – Effects of CYR61 downregulation and ZOL addition on Ras→Raf and Akt-dependent pathways on PC3 cells. Parental or shCYR61-transfected PC3 cells were treated with 50 μM ZOL for 24 hr. Thereafter, both the expression and activity of Erk and Akt and the expression of cell cycle inhibitors p21 and p27 were evaluated. Determination of the expression *a)* and phosphorylation *b*) of Erk-1/2 evaluated after blotting with specific antibodies, as described in ''Material and Methods''. Evaluation of the expression *c*) and activity *d*) of Akt analyzed after blotting with an anti-Akt and an anti-pAkt specific antibodies, respectively, as described in ''Material and Methods''. Expression of p21 *e*) and p27 *f*) evaluated after blotting with specific Mabs. *h*) Expression of the house-keeping protein α-tubulin, used as loading control. The experiments were performed at least 3 different times, and the results were always similar. CTR: untreated PC3 cells; LIPO: PC3 cells exposed to lipofectamine for 24 hr; Sc: PC3 cells transfected with scrambled vector and cultured for 24 hr without ZOL; Sc/ZOL: PC3 cells transfected with scrambled vector and exposed for 24 hr to 50 μM ZOL; shCYR61: PC3 cells transfected with shCYR61 and cultured for 24 hr; shCYR61/ZOL: PC3 cells transfected with shCYR61 and exposed to 50 μM ZOL for 24 hr.

evaluated by FACS analysis, whereas the effects of ZOL on CYR61 expression of parental PC3 cells were mild (Fig. 4*b*) as expected since the maximal regulation of CYR61 expression occurred at 48 hr.

We have treated parental and transfected cells with ZOL and evaluated the effects on cell growth inhibition and cell cycle distribution. The transfection with the shCYR61 potentiated the antitumor effects induced by ZOL reaching about 80% of growth inhibition when transfected PC3 cells were treated with 100 μM of ZOL for 24 hr that, on the other hand, caused only an about 65% growth inhibition in parental PC3 cells (Fig. 4*c*). Notably, 10 μM ZOL induced only 2% growth inhibition in parental cells but the same treatment caused an about 20% growth arrest in transfected cells with a consequent potentiation factor of about 2.5-fold. On the other hand, the transfection alone with either the scrambled vector or shRNA for CYR61 was not able to induce significant effects on growth inhibition (Fig. 4*c*).

Downregulation of CYR61 induced by shCYR61 increased the percentage of cells in S-phase (17% *vs.* 6% of parental cells) but did not change cell distribution in G_2/M phase (22% *vs.* 23% of parental cells) (Fig. 4*d*). The treatment of transfected PC3 cells with 50 μM ZOL for 48 hr potentiated the effects on S-phase (25%) and decreased cell accumulation in G_2/M phase (13%) suggesting a retardation of the progression of PC3 cells from S-phase to G_2/M phase (Fig. 4*d*).

These data suggest an involvement of CYR61 in the regulation of cell growth and cell cycle distribution of PC3 cells treated with ZOL.

Effect of shCYR61 and ZOL on Ras-dependent signal transduction pathway and on cell cycle inhibitors of PC3 cells

ZOL is an aminobisphosphonate that inhibits the farnesyl pyrophosphate synthase essential for small G-proteins prenylation, and, thus, it is able to prevent the Ras→Raf-dependent signaling. As expected and previously reported,[20] 48 hr 50 μM ZOL induced an about 1.5-fold decrease of both Erk-1/2 and Akt activity. Moreover, shCYR61 transfection induced an about 1.5-fold decrease of Akt activity, whereas Erk-1/2 activity was almost unchanged (Figs. 5*b* and 5*d*). The downregulation of CYR61 synergized with ZOL on the inhibition of Erk-1/2 and Akt activity. In fact, an about 2.5-fold decrease of the activity of the two enzymes was recorded in ZOL-treated transfected PC3 cells (Figs. 5*b* and 5*d*) without affecting both Erk-1/2 and Akt expression (Figs. 5*a* and 5*c*). On the other hand, either the transfection with empty vector or the addition of lipofectamine did not induce any change of enzyme activity (Figs. 5*b* and 5*d*). Moreover, we have studied the changes in the expression of cell cycle inhibitors $p21^{waf1}$ and $p27^{kip1}$ after transfection with shCYR61 and treatment with ZOL. Both the downregulation of CYR61, obtained with use of shRNA for CYR61, and the treatment with 50 μM ZOL for 48 hr induced an about 2-fold increase of p21 expression that was potentiated by ZOL addition to transfected cells (about 3.5-fold increase of the protein expression) (Fig. 5*e*). Moreover, p27 was not modulated by ZOL and 1.5-fold increase was induced by the transfection with shCYR61, whereas the treatment of shCYR61-transfected cells with ZOL caused an about 2.5-fold increase of the protein (Fig. 5*f*). These findings, at least in part, explains the modification of cell cycle induced by shCYR61 and/or ZOL.

Effects of CYR61 on motility and invasiveness of cells treated with ZOL

Since CYR61 is a ligand of αvβ3 integrin we have evaluated the effects of CYR61 knock down on motility and invasiveness of PC3 cells.

We have found that both downregulation of CYR61 and treatment with 50 μM ZOL for 48 hr produced an about 40–50% reduction of cell motility in Boyden chambers (Figs. 6*a* and 6*c*) that was strongly potentiated in transfected cells treated with ZOL reaching an about 75% inhibition (Figs. 6*a* and 6*c*). We found that untreated PC-3 cells migrated through Matrigel matrix membrane more than cells transfected with shCYR61 or treated with ZOL where the invasion was 10–20% inhibited (Figs. 6*b* and 6*c*). Downregulation of CYR61 in cells treated with ZOL slightly increased the latter effect (about 35%) (Figs. 6*b* and 6*c*). These results demonstrate that CYR61 is involved in invasion in human androgen-independent prostatic cancer cell lines and its downregulation can increase the effects induced by ZOL on prostate cancer cell motility and, at a less extent, invasion.

Discussion

Prostate cancer is the most common malignancy found in men. Although prostate cancer cells are represented widely in the peripheral circulation of men with advanced disease,[29] metastatic deposits tend to preferentially occur in the bone resulting in 84% of men dying and are directly responsible for considerable morbidity in the time to death.[30–32] In the marrow, the prostate epithelial cells bind rapidly to the bone marrow endothelium by an integrin-mediated process.[33] Once bound, they migrate through the endothelial layer, following which, some of them go on to form metastases. The process by which this occurs is poorly understood but it is known to involve a combination of enhanced cell motility and degradation of the basement membrane by CYR61 and degradative enzymes such as matrix metalloproteinases.[34,35]

CYR61 has been reported to participate in the development and progression of several cancers and was described recently to be involved both in benign prostatic enlargement[36] and in growth, migration and metastasis of prostate cancer cells.[35]

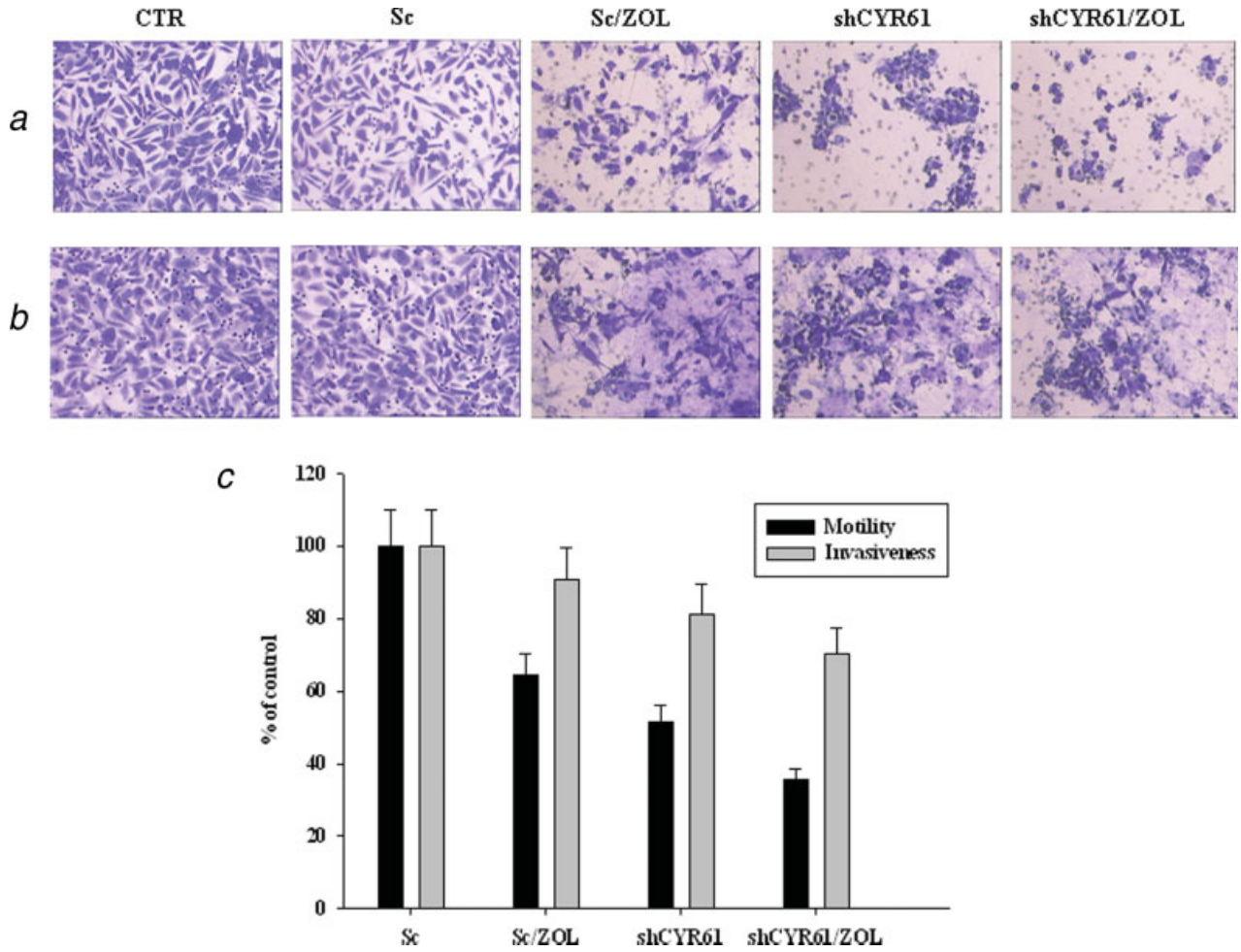


FIGURE 6 – Role of CYR61 modulation on motility and invasiveness of cells treated with ZOL *a*) PC3 parental or transfected cells were plated in the top chamber of noncoated polyethylene teraphthalate (PET) membranes, treated or not with 50 μM ZOL for 48 hr as described in ''Material and Methods'', and cell motility was evaluated. The migrated cells were stained with 0.25% crystal violet for 10 min and photographed under the microscope. *b*) For *in vitro* invasion assays, PC3 cells were added to a Boyden chamber coated with Matrigel as described in ''Material and Methods'', and the cell invasion was evaluated. The invaded cells were stained with 0.25% crystal violet for 10 min and photographed under a microscope. *c*) The number of migrated and invaded cells were quantified with a spectrophotometer as OD, and the results are expressed as a percentage of PC3 parental cells. The experiments were performed at least 3 different times, and the results were always similar. Bars, SDs. CTR: untreated PC3 cells; LIPO: PC3 cells exposed to lipofectamine for 48 hr; Sc: PC3 cells transfected with scrambled vector and cultured for 48 hr without ZOL; Sc/ZOL: PC3 cells transfected with scrambled vector and exposed for 48 hr to 50 μM ZOL; shCYR: PC3 cells transfected with shCYR61 and cultured for 48 hr; shCYR/ZOL: PC3 cells transfected with shCYR61 and exposed to 50 μM ZOL for 48 hr. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The bisphosphonate class of drugs has been shown to be of therapeutic benefit in reducing skeletal morbidity from bone metastases in breast cancer[37] and more recently, ZOL, the most potent of these agents, has been proven to reduce morbid skeletal related events in prostate cancer.[32] This has been hypothesized to be due to reduction in the osteoclast-mediated bone resorption known to be associated with this disease.[38,39] *In vitro* studies have demonstrated that the binding of breast and prostate cancer to bone surfaces is inhibited by ZOL,[40] and the same group has suggested that this drug also has an inhibitory effect on cell proliferation. In experiments using prostate cancer cell lines, a decrease of cellular migration was observed when cells were cultured with ZOL.[13] Similar effects have been obtained with ZOL in breast cancer, and it has been suggested that this mechanism is mediated by the effects of the drug acting on the cytoskeleton through a RhoA-mediated mechanism and by inhibition of specific chemokines known to be involved in the development of bone metastases (such as stromal derived growth factor-1, SDF-1).[41]

We have demonstrated previously that ZOL induces a time and dose-dependent growth inhibition and apoptosis of human androgen-independent PC3 and DU145 and androgen-dependent LnCaP prostate adenocarcinoma cell lines. Moreover, ZOL interferes with the mevalonate biosynthetic pathway, and affects cell activity and survival by interfering with protein prenylation and, therefore, the signaling functions of key regulatory proteins belonging to Ras→Erk pathway. However, ZOL has pleiotropic effects on the modulation of activity and/or expression of different intracellular proteins involved in the regulation of transformed phenotype, and the molecular targets responsible for the anticancer effect of the drug are still unknown. On the basis of these considerations, we have performed a gene expression profiling of androgen-independent PC3 cancer cells treated with ZOL, and we have demonstrated for the first time, at least at our knowledge, that ZOL strongly downregulates CYR61, both at mRNA and protein levels. Moreover, the transcriptional activity of CYR61 promoter evaluated through a luciferin–luciferase assay resulted decreased in ZOL-treated PC3 cells in a dose- and time dependent manner (with a peak at 12 hr).

Interestingly, the downregulation of CYR61 induced by ZOL treatment was paralleled by the block of the Ras→Raf-dependent

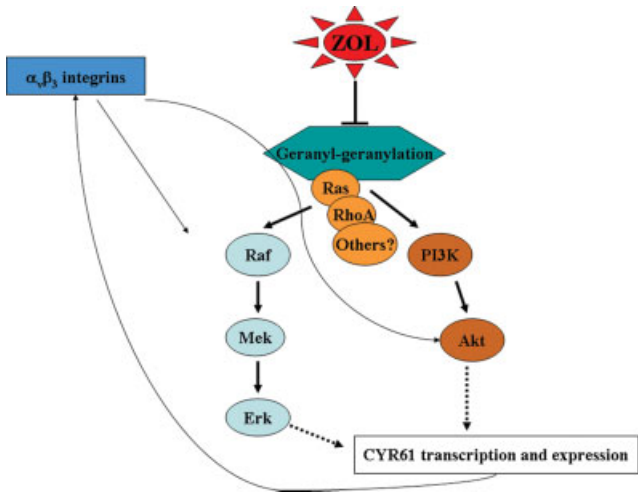


FIGURE 7 – Schematic representation of the regulation of CYR61 expression by ZOL. ZOL inhibits isoprenylation (above all geranyl-geranylation) of small GTP binding proteins (*i.e.*, Ras and/or RhoA and/or others) and thus, blocks the Ras→Raf→Mek→Erk-dependent or the PI3K→Akt-mediated pathways. The latter effects block the transcription of the CYR61 gene and consequently of the related protein. The lack of CYR61 expression reduces the activation of the integrin-dependent pathways potentiating the inhibition of Erk and Akt activities (dashed arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pathway and both effects were dependent from isoprenylation inhibition. In fact, the effects of ZOL were antagonized partially or completely by the addition of either FOH or GGOH, respectively, to treated PC3 cells. It is noteworthy that the abrogation of the effects induced by ZOL was complete only with GGOH. These results can be explained by the presence in PC3 cells of Ki-Ras and N-Ras isoforms that can be both granylgeranylated and farnesylated,[42] and, in addition, the geranylgeranylation could have functions different from those of farnesylated isoforms. Moreover, another target of ZOL is RhoA that is geranylgeranylated and involved in the regulation of cell adhesion, a process regulated by CYR61.[42] Interestingly, CYR61 modulation was induced specifically by ZOL but not at all or only partially by other specific inhibitors of the Ras→Raf-dependent pathway such as R115777, gefitinib, LY294002 or BAY 43-9006 suggesting the following conclusions: *i*) the inhibition of farnesylation is not sufficient to induce regulation of CYR61 expression; *ii*) EGF-R is not a critical regulator of CYR61 expression, at least, in prostate cancer cells; *iii*) both Ras→Erk-dependent and Akt pathways equally contributed to CYR61 downmodulation (Fig. 7). These findings agreed partially with findings demonstrating that CYR61 expression is negatively and transcriptionally regulated by FOXO3A that is inhibited by Akt.[43,44] Although Akt is a major regulator of FOXO3A, serum/glucocorticoid regulated kinase and IkB kinase also phosphorylate and inactivate FOXO3A[45,46] suggesting a concomitant involvement of other intracellular kinases such as Erk in the regulation of FOXO3A activity. Finally, the cytotoxic drug DTX had opposite effects increasing CYR61 expression and suggesting a specific involvement of signal transduction pathways in its modulation. Interestingly, we and others have demonstrated that IC_{50} concentrations of DTX induced an escape mechanism from antiproliferative effects based on the overactivation of a Ras→Erk-dependent pathway in human epithelial cells including prostate cancer cells.[47,48] The overactivation of these survival pathways could explain partially the increase of CYR61 expression induced by DTX.

Thereafter, we have investigated on the role of CYR61 in growth regulation of PC3 cells using a shRNA for CYR61 to downregulate the expression of the protein. The transfection of PC3 cells with shCYR61 increased the growth inhibition induced by low doses of ZOL with a potentiation factor of 2.5, whereas the transfection of PC3 cells with shCYR61 alone was ineffective. The potentiation of growth inhibition induced by ZOL in cells lacking CYR61 occurred together with a decrease of cells in the G2/M phase of the cell cycle. The latter effect was paralleled by an increase of p21 and p27 cell cycle inhibitors expression that was again potentiated in shCYR61-transfected cells treated with ZOL.

Since CYR61 is a ligand of αvβ3 integrin, we have evaluated the effects of CYR61 knock down on motility and invasion of PC3 cells. Cell motility inhibition induced by downregulation of CYR61 expression was potentiated strongly by ZOL. CYR61 downregulation is involved in the regulation of motility processes and, indirectly, of cell growth inhibition induced by ZOL.[26] In fact, it has been demonstrated that both the Ras-dependent Erk and the Akt-mediated pathways can be activated and driven the transformed and apoptosis-resistant phenotype of cancer cells consequently to the binding of CYR61 to αvβ3 integrins.[49] On the basis of this and previous results, it could be interesting to develop molecular therapeutic strategies based on the specific interruption of this loop through the inhibition of the expression and/or function of CYR61. Therefore, we are evaluating the activity of blocking anti-CYR61 antibodies in inducing the potentiation of ZOL antiproliferative and anti-invasive effects in human prostate cancer cells.

In conclusion, CYR61 appears a suitable additional target to enhance the antitumor effects of ZOL and the use of DNA microarray has demonstrated its ability in searching for additional molecular targets of antitumor agents currently used in anticancer therapy. These data strongly encourage the design of clinical trials based on the concomitant administration of ZOL and anti-CYR61 antibodies in patients with CYR-61 overexpressing cancers.

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