

Research paper

Ferruginol suppresses survival signaling pathways in androgen-independent human prostate cancer cells

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Abstract

Ferruginol, a bioactive compound isolated from a Chilean tree (Podocarpaceae), attracts attention as a consequence of its pharmacological properties, which include anti-fungal, anti-bacterial, cardioprotective, anti-oxidative, anti-plasmodial and anti-ulcerogenic actions. Nevertheless, the molecular basis for these actions remains only partly understood and hence we investigated the effects of ferruginol on androgen-independent human prostate cancer cells (PC3), a known model for solid tumor cells with an exceptional resistance to therapy. The results show that ferruginol induces PC3 cell death via activation of caspases as well as apoptosis-inducing factor (AIF) as confirmed by its translocation into the nucleus. In order to clarify the biochemical mechanism responsible for the anti-tumor activity of ferruginol, we analyzed a set of molecular mediators involved in tumor cell survival, progression and aggressiveness. Ferruginol was able to trigger inhibition/downregulation of Ras/PI3K, STAT 3/5, protein tyrosine phosphatase and protein kinases related to cell cycle regulation. Importantly, the toxic effect of ferruginol was dramatically impeded in a more reducing environment, which indicates that at least in part, the anti-tumoral activity of ferruginol might be related to redox status modulation. This study supports further examination of ferruginol as a potential agent for both the prevention and treatment of prostate cancer.

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Keywords: Natural compound; Ferruginol; Apoptosis; Prostate cancer

Abbreviations: AIF, apoptosis-inducing factor; Bax, Bcl-2 associated x protein; CDKIs, cyclin-dependent kinase inhibitors; CDKs, cyclin-dependent kinases; DISC, death-inducing signaling complex; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated protein kinase 1/2; GSK-3 β , glycogen synthase kinase-3 β ; Hsp27, heat shock protein 27; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; p21, tumor suppressor protein; PC3, androgen-independent prostate cancer cell line; PI3K, phosphoinositide 3-kinase; Rb, retinoblastoma; STAT, signal transducer and activator of transcription; TNFR, tumor necrosis factor receptor; FADD, Fas-associated death domain protein; AKT/PKB, protein kinase B; PCNA, proliferating cellular nuclear antigen; NF κ B, nuclear factor kappa B; IKK, inhibitory-kappa B kinase; LMWPPT, low molecular weight protein tyrosine phosphatase; JAK, Janus kinase.

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1. Introduction

Prostate cancer is a major cause of cancer-related death among males and the second leading cause of cancer death in Western countries. Although recent years have seen an improvement in prostate cancer diagnosis, only a few novel therapeutic strategies have emerged and there has been little progress in improving survival [1,2]. Therefore, novel strategies for dealing with this disease are called for. Our research group has a long-standing interest in the possible beneficial biological effects of natural compounds and/or their derivatives, such as antioxidants [3] and anti-tumor agents [4–10], and hence we were interested whether we could define novel compounds with therapeutic potential for prostate cancer. Among the different classes of natural compounds, the diterpenoids have been shown to present a potent anti-proliferative action [4,11]. Ferruginol, an active compound isolated from the Chilean tree *Persea nubigena* and from the stem bark of *Podocarpus andina* (Podocarpaceae), is an abietane diterpene occurring in plants belonging to the Podocarpaceae, Cupressaceae, Lamiaceae and Verbenaceae families. This diterpene presents promising biological activities, such as anti-fungal and anti-bacterial [12], mitocidal [13], cardioactive [14], anti-oxidative [15], anti-plasmodial [16] and anti-ulcerogenic [17] properties.

We decided to investigate the potential effects of ferruginol in prostate cancer. In this work we show for the first time the molecular mechanism by which ferruginol, induces resistant prostate cancer cell death. Ferruginol was able to trigger inhibition/downregulation of Ras/PI3K, STAT 3/5, protein tyrosine phosphatase and protein kinases related to cell cycle regulation. Importantly, the toxic effect of ferruginol was dramatically impeded under the condition of more reducing environment, which indicates that at least in part, the anti-tumoral activity of ferruginol might be related to redox status modulation. This study supports further examination of ferruginol as a potential agent for both the prevention and treatment of prostate cancer.

2. Material and methods

2.1. Cell line and reagents

PC3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Ferruginol (Fig. 1A) was extracted from the wood of *P. nubigena* Lind. and from the stem bark of *P. andina* (Poepp. ex Endl.) de Laub. (Podocarpaceae) as previously described [18].

Polyclonal antibodies against phospho p38MAPK, phospho-p42/p44 MAPK (ERK1/2) Thr202/204, ERK1/2, phospho-MEK1/2 Ser217/221, pan-AKT, phospho-AKT Ser473, phospho-Hsp27 Ser82, phospho-c-Raf Ser338, phospho-GSK-3 β Ser9, AIF, phospho-cdc2 Thr15, phospho-Rb Ser795, phospho-STAT5 Tyr694, phospho-PI3K p85 subunit, CDK6, CDK4, cyclin D1, cyclin D3, PCNA, tubulin, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology

(Beverly, MA). Antibodies against p21, NF κ B p65 subunit, phospho-STAT3 Tyr705, phospho-STAT3 Ser727, Bcl2, Bax, TNF receptor 1, FADD, IKK α and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LMWPTP antibody was from Abcam. Caspases 3, 8 and 9 Colorimetric Assay Kits were obtained from R&D Systems (Minneapolis, MN).

2.2. Cell culture

PC3 cells were cultured in RPMI containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum, at 37 °C in a 5% CO₂ humidified atmosphere. In all experiments, cells at semi-confluence were treated for 24 h with different concentrations of ferruginol.

2.3. MTT assay for cellular viability

Cell viability was assessed by MTT reduction assay as previously reported [19,20]. The effect of ferruginol on cell growth was assessed as the percentage of inhibition in cell growth where non-treated cells were taken as 100% of viability. IC₅₀ values were determined from three independent experiments.

2.4. Western blotting analysis

Following treatment of cells with ferruginol, the medium was aspirated and the cells were washed with cold physiological solution. The cells were then incubated in 200 μ l of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na₃VO₄, 0.25% sodium deoxycholate and protease inhibitors (1 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride)) over ice for 30 min. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Lowry method [21]. An equal volume of 2 \times sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 50 μ g of protein, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (1%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4 °C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. The detection was made using enhanced chemiluminescence ECL.

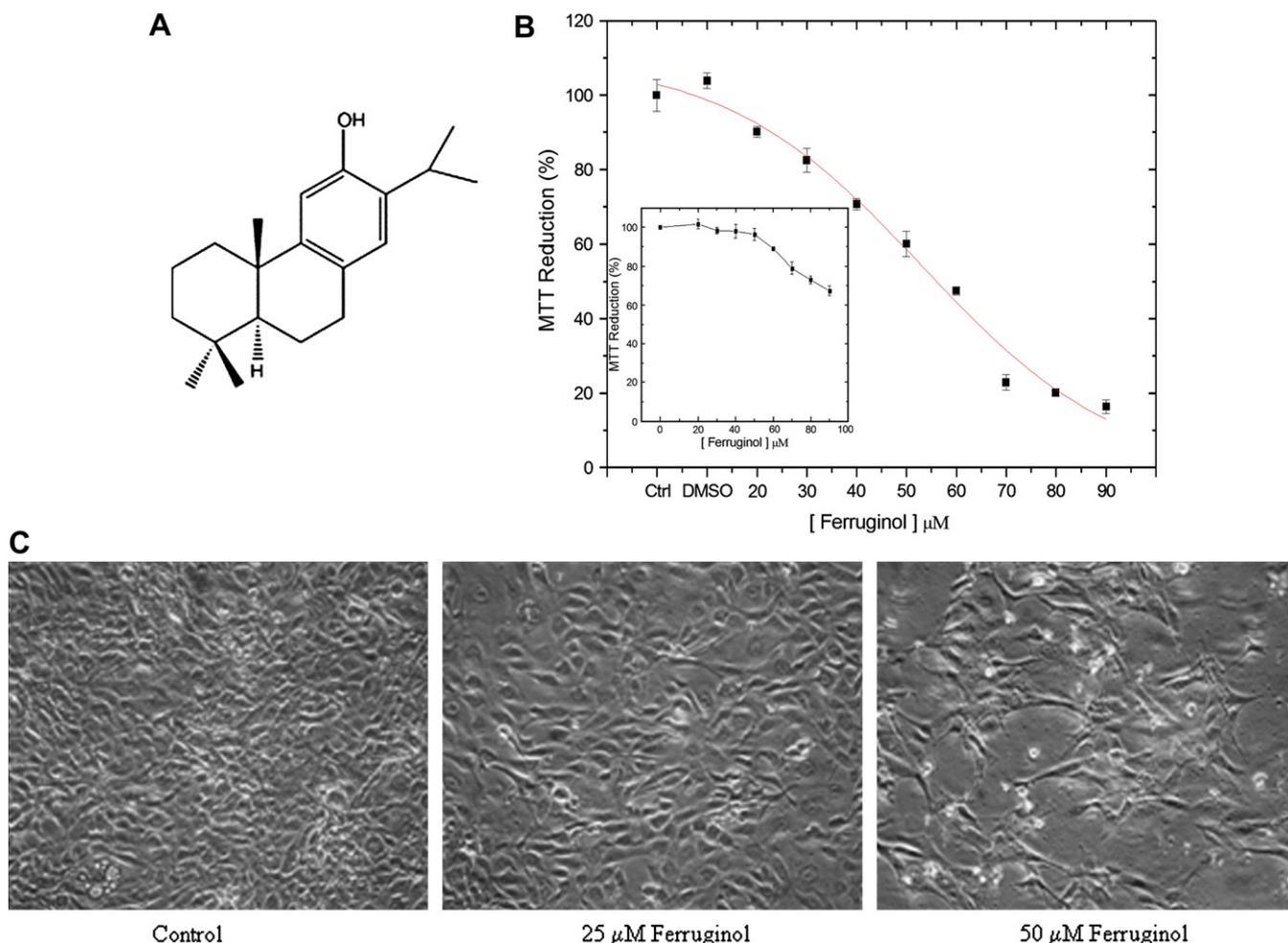


Fig. 1. Ferruginol induces toxic effects in PC3 cells. Cells were treated with specified concentrations of ferruginol (A) for 24 h. (B) Mitochondria function was evaluated through the MTT reduction. The results are expressed as the mean % of absorbance (ratio of absorbance in ferruginol treated and control cells); cell viability was also evaluated when the cells were pre-treated for 1 h with 10 mM GSH (inset plot). (C) Morphological analysis of PC3 cells treated with 25 μM and 50 μM ferruginol for 24 h (100× magnification). Ferruginol was dissolved in DMSO and the final concentration of this solvent was remained at 0.1%.

2.5. Immunoprecipitation of LMWPTP

After treatment of the cells with ferruginol for 24 h, whole-cell lysates were prepared with lysis buffer (20 mM HEPES pH 7.7, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1% Nonidet-P40 (NP40), 1 mM 4-(2-amino-ethyl)-benzenesulfonylfluoride hydrochloride), 1 mM DTT, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and chilled on ice for 2 h. After centrifugation, lysates were rotated with anti-LMWPTP and Protein A–Sepharose at 4 °C for 2 h. The beads were washed three times with lysing buffer and twice with 0.5 M Mes, pH 6.0. Next, the phosphatase activity was determined using pNPP as a substrate.

2.6. Caspases 3, 8 and 9 activity assays

Caspase activities were determined by the measurement at 405 nm of *p*-nitroaniline (pNA) released from the cleavage of Ac-DEVD-pNA, IETD-pNA and LEHD-pNA as substrates of caspases 3, 8 and 9, respectively. The enzyme activities were

expressed in pmol/min and the extinction coefficient of pNA was $10,000 M^{-1} cm^{-1}$.

2.7. NFκB p65 and AIF nuclear translocation

Briefly, 2×10^7 cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.2 ml ice-cold cell extract buffer (10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethsulfonyl fluoride (PMSF)). The cells were kept on ice for 10 min to allow them to swell, mixed by vortex for 10 s, and microfuged at 4 °C at $14,000 \times g$ for 30 s. The supernatant was discarded, and the pellet was resuspended in 30 μl nuclear extraction buffer (20 mM HEPES–KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA (ethylenediaminetetraacetic acid), 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 min, and centrifuged at 4 °C at $14,000 \times g$ for 2 min. The supernatant was saved as the nuclear extract and used in Western blotting assay.

2.8. Quantification of reduced and oxidized glutathione

Cells were washed twice with PBS, detached with 5 mM EDTA in PBS and washed twice again with cold PBS. Cell number was counted using a hemocytometer; afterwards the cells were centrifuged and equal volumes of cold PBS and 6% 5-sulfosalicylic acid (SSA) were added to cell pellets. The samples were sonicated, centrifuged at 10,000 rpm for 5 min and the supernatants used for the assays. Total glutathione (GSH + GSSG) and glutathione disulfide (GSSG) were determined by using recycling assays involving the reaction of 5,5'-dithio-bis(2-nitrobenzoic acid) and glutathione reductase. The total amount of glutathione was calculated from a reduced glutathione standard curve prepared in SSA. For GSSG assay, 100 μ l of supernatant was incubated with 2 μ l of 2-vinylpyridine for 60 min on ice. The amount of GSSG was calculated from the GSSG standard curve. The amount of reduced GSH per cell was calculated by subtracting the amount of GSSG per cell from the amount of total glutathione per cell [22].

2.9. Annexin V and 7-amino-actinomycin D assays

Control and ferruginol-treated cells were collected and resuspended in $1 \times$ binding buffer (0.01 M HEPES–NaOH (pH 7.4), 0.14 mM NaCl and 2.5 mM CaCl₂) at a concentration of 2×10^7 cells/ml. Subsequently, 100 μ l of cell suspension was transferred to a 5 ml tube and 5 μ l each of Annexin V-APC and 7-amino-actinomycin D (7-AAD) was added. Cells were incubated at room temperature for 15 min, after which 400 μ l of $1 \times$ binding buffer was added and apoptosis detected by flow cytometry (Becton Dickinson FACSCalibur, Rockville, MD), the data obtained were analyzed using the software Cell Quest Pro BD Biosciences Pharmingen (Erembodegem, Belgium).

2.10. Flow cytometry

After treatment of PC3 cells with ferruginol for 24 h, cells were harvested by the addition of 5 mM EDTA and gently washed off the plate. Cells were pelleted along with the previously collected media. Cell pellets were fixed with 70% ethanol for 30 min on ice and then rinsed three times with 1 ml of 0.1% glucose in PBS (20 mM NaH₂PO₄, 150 mM NaCl), pelleted and resuspended in propidium iodide (PI) staining solution (10 μ l of 10 mg/ml RNase A, 5 μ l of 10 mg/ml PI per 1 ml of PBS with 0.1% glucose). After 30 min the cells were analyzed using a flow cytometer.

2.11. Statistical analysis

All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Cell viability data were expressed as the mean \pm standard error of three independent experiments carried out in triplicate. Data from each assay were analyzed statistically by ANOVA. Differences were considered significant when the *P* value was

less than 0.05. Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning the film (data are presented as the protein to β -actin or tubulin ratio).

3. Results

3.1. Inhibition of PC3 growth by ferruginol

PC3 cells were treated with ferruginol in concentrations up to 100 μ M and the effect of ferruginol on cell viability was determined employing the MTT method. As shown in Fig. 1B, ferruginol caused a dose-dependent reduction in the cell number displaying an IC₅₀ value of 55 μ M. Importantly, pre-treatment of the cells with 10 mM GSH prevented the toxic action of ferruginol (inset plot). In agreement, the microscopy analysis also demonstrated a decrease of the cell number (Fig. 1C).

3.2. Ferruginol induces apoptosis of PC3 cells via caspases and AIF activation

In the next series of experiments, it was determined whether treatment of PC3 cells to ferruginol led to apoptosis. Ferruginol caused around 15% and 30% (in the presence of 25 and 50 μ M ferruginol, respectively) of cell death via apoptosis as detected through phosphatidylserine exposure (Fig. 2A). In agreement, we also observed activation of caspases 8, 9 and 3 (Fig. 2B). Additionally, at the lowest concentration, ferruginol led to an overexpression of TNFR1; however, FADD expression was not affected. We also examined the possible participation of mitochondria in response to ferruginol. Bcl2:Bax ratio was not significantly affected; however the expression of AIF was dramatically increased at 50 μ M ferruginol. Accordingly, AIF nuclear translocation was detected (Fig. 2C). These findings suggest that PC3 cell response toward ferruginol involves activation of caspases and release of AIF from mitochondrial intermembrane space.

3.3. Ferruginol treatment impairs prostate cancer cell survival

To obtain more insight into the molecular mechanisms mediating ferruginol effects on PC3 cells, the phosphorylation/expression state of a panel of signal transduction mediators in response to ferruginol was examined. As shown in Fig. 3, PC3 cell treatment with 50 μ M ferruginol provoked downregulation of p85 subunit of PI3K and inhibition of AKT. The results presented above indicate that ferruginol should produce a decrease of the survival and anti-apoptotic relevant kinase activities. Accordingly, cells treated with 50 μ M ferruginol displayed activation of MAPK p38, an important apoptosis inducer, and a slight inhibition of ERK2. However, the upstream activator of ERK, MEK, was not affected. Apparently, ferruginol impairs prostate cancer proliferation by modulating survival and proliferation signaling cascades.

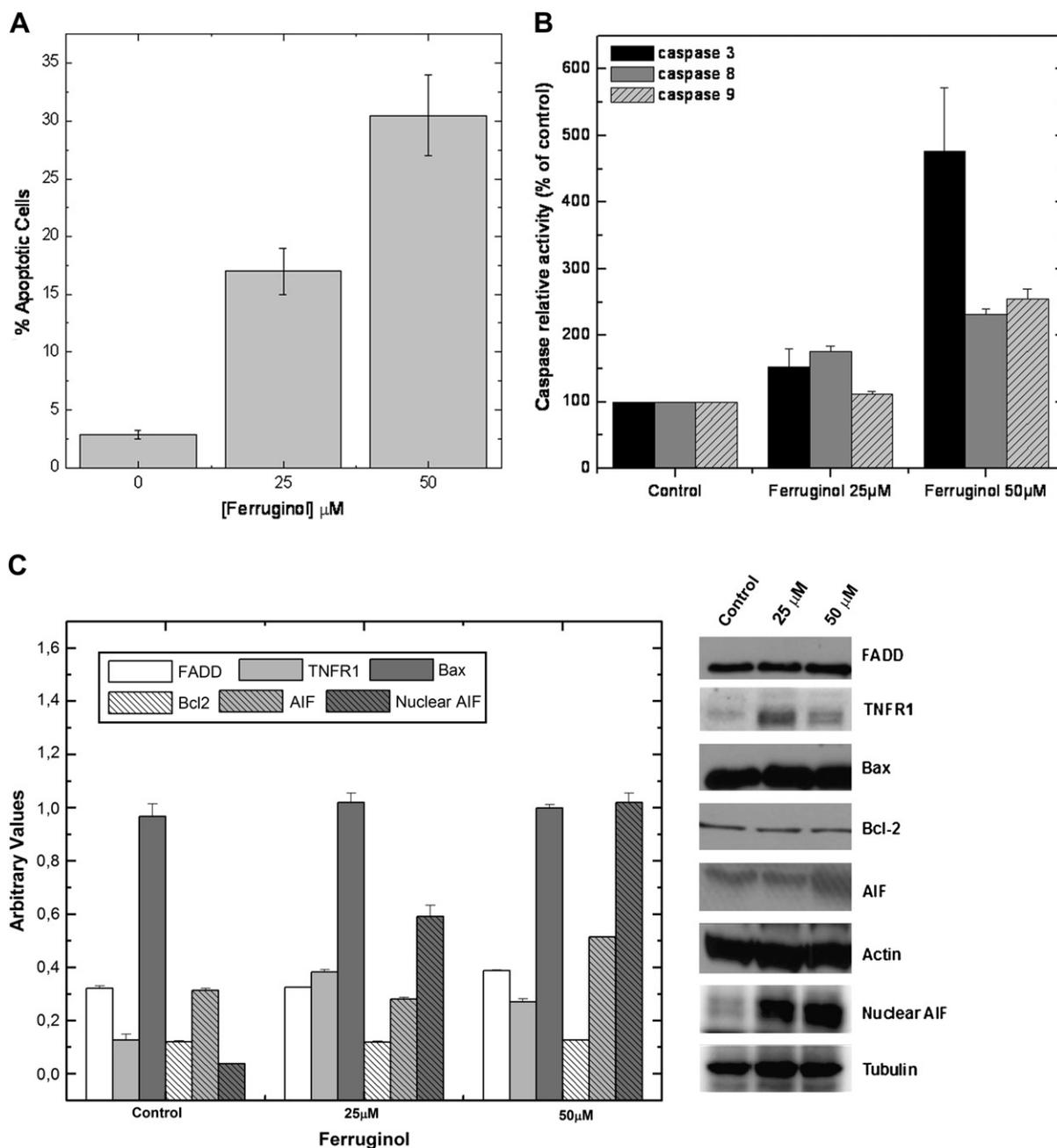


Fig. 2. Apoptosis induction of PC3 cells by ferruginol. (A) Cell samples were prepared as described in Section 2 and Annexin V-positive, 7-AAD-positive and Annexin V/7-AAD-positive populations were analyzed by flow cytometry. (B) Caspases 3, 8 and 9 activities were determined by using colorimetric assay. (C) The expression of pro- and anti-apoptotic proteins was determined by Western blot analysis. Soluble lysates were matched for protein content and analyzed on Western blot and immunoblots were probed with actin antibody to ensure equal loading. Nuclear translocation of AIF was also determined.

3.4. Inhibition of cell cycle progression by ferruginol

By plotting the ratio of the cells in G_0 plus G_1 against cells in G_2/M plus S-phase it is apparent that PC3 cells undergo G_0/G_1 -phase cell cycle arrest after ferruginol treatment (Fig. 4A). Approximately 2.5-fold of the PC3 population was at phase G_0/G_1 after exposure to ferruginol for 24 h.

We therefore turned to characterize the effect of ferruginol treatment on direct regulators of cell cycle progression. As shown by immunoblot analysis (Fig. 4B), the expression of

p21 was increased even at the lowest concentration of ferruginol. On the other hand, the phosphorylated Rb protein and the expression of PCNA were not affected. The level of phospho-cdc2, the key protein of the cell cycle progression from G_2 to M phase, remained unchanged. Ferruginol decreased the level of CDK4, CDK6, cyclin D1 and cyclin D3. This response directly mirrored the ability of ferruginol to induce cell cycle arrest in PC3 cells. We conclude that the combination of reduced mitotic activity and induction of apoptosis accounts for the observed cytotoxic effect of ferruginol on PC3 cells.

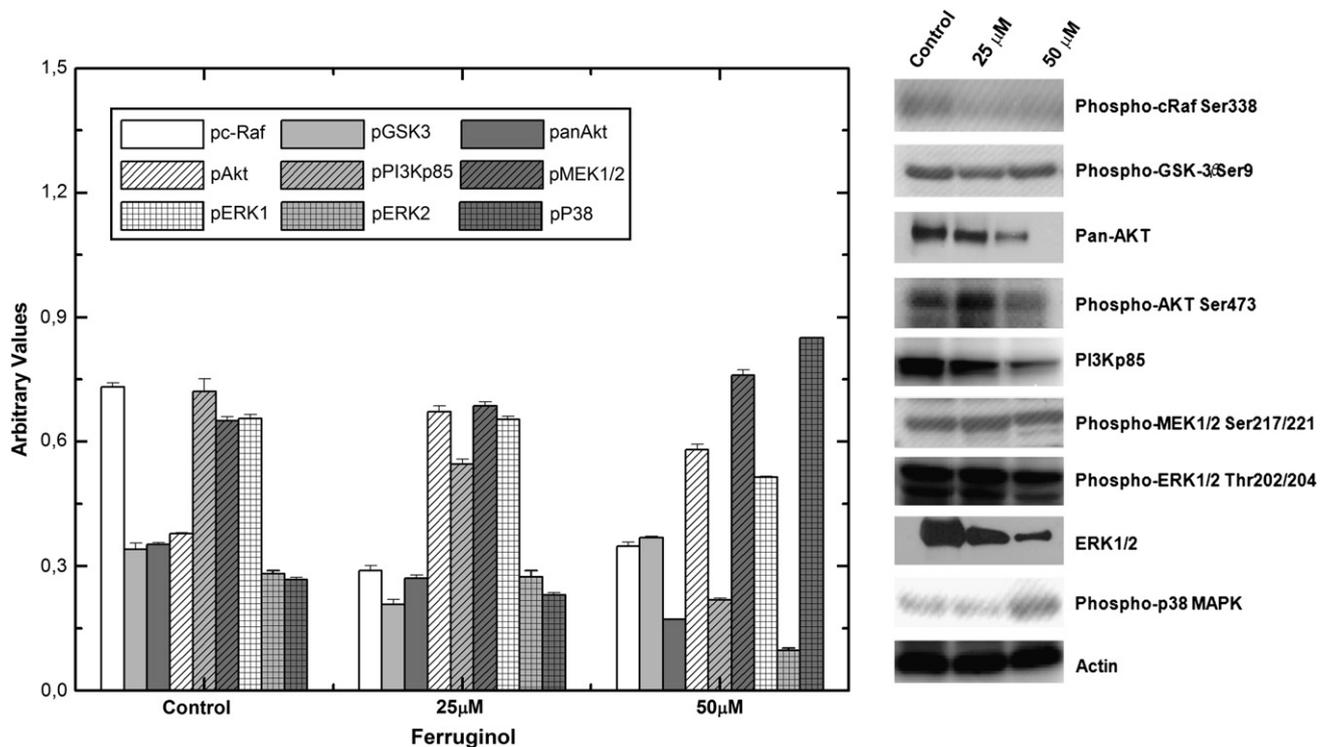


Fig. 3. Effect of ferruginol treatment on the function of key mediators involved in the PC3 cells proliferation/survival. Cells were treated with specified concentrations of ferruginol for 24 h and the expression or phosphorylation of the proteins determined by Western blot. Equal loading was confirmed by reprobing them for β -actin.

3.5. Ferruginol causes downregulation of *IKK α* and hypophosphorylation of *STATs*

The immunoblot analysis data showed that the expression of *IKK α* was significantly decreased after treatment with ferruginol; however, the total level of *NF κ B* remained unchanged (Fig. 5). In addition, ferruginol caused a decrease of this transcription factor into the nucleus.

The phosphorylation status of *STAT 3* and *5*, key signaling molecules for many cytokines and growth-factor receptor response, was also evaluated by Western blot. The treatment of PC3 with 50 μ M ferruginol was able to decrease the phosphorylation levels of *STAT3* (Tyr705) and *STAT5* (Tyr694). Interestingly, 50 μ M ferruginol caused a strong decline in *Hsp27* expression, which can be associated with the increase in the number of apoptotic cells.

3.6. Redox status on PC3 cells treated with ferruginol

Based on the diterpene chemical properties, which can lead to antioxidant and/or oxidant action depending on its concentration, and the importance of reducing equivalents for PC3 cells survival [23], we investigated the effect of ferruginol on PC3 cell GSH metabolism. PC3 cells treated with ferruginol displayed a more oxidizing environment as defined by a decrease of GSH and an increase of GSSG levels (Fig. 6). Both effects were dose dependent and the highest concentration of ferruginol employed caused a 2-fold change. It is important to note that even at the highest concentration of ferruginol, GSH:GSSG

ratio remained almost in equilibrium (GSH:GSSG = 4.4, 2.7 and 1.1 at 0, 25 and 50 μ M ferruginol, respectively).

3.7. Low molecular weight protein tyrosine phosphatase is modulated by ferruginol

Besides the fact that protein tyrosine phosphatases are highly sensitive to cell redox status, there is some evidence that this class of phosphatases can contribute to tumor cell progression and aggressiveness. Especially LMWPTP has been recognized as a positive regulator of tumor growth [24]. Therefore, to address the possible modulation of LMWPTP by ferruginol, we examined the LMWPTP activity as well as expression. LMWPTP immunoprecipitated from PC3 cells was inhibited around 30% by 50 μ M ferruginol (Fig. 7). On the other hand, when the LMWPTP activity was checked after treating the cells for 24 h, this enzyme displayed only 20% of residual activity. Reduced LMWPTP activity is consistent with the change in redox status in response to ferruginol. In addition, treated cells demonstrated downregulation of LMWPTP expression.

4. Discussion

Prostate cancer is commonly malignant and it is the second leading cause of cancer-related deaths (after lung cancer) of males in Brazil, with a similar trend in many Western countries (data from Instituto Nacional do Câncer—INCA). Since prostate cancer usually occurs in men aged 50 years and older

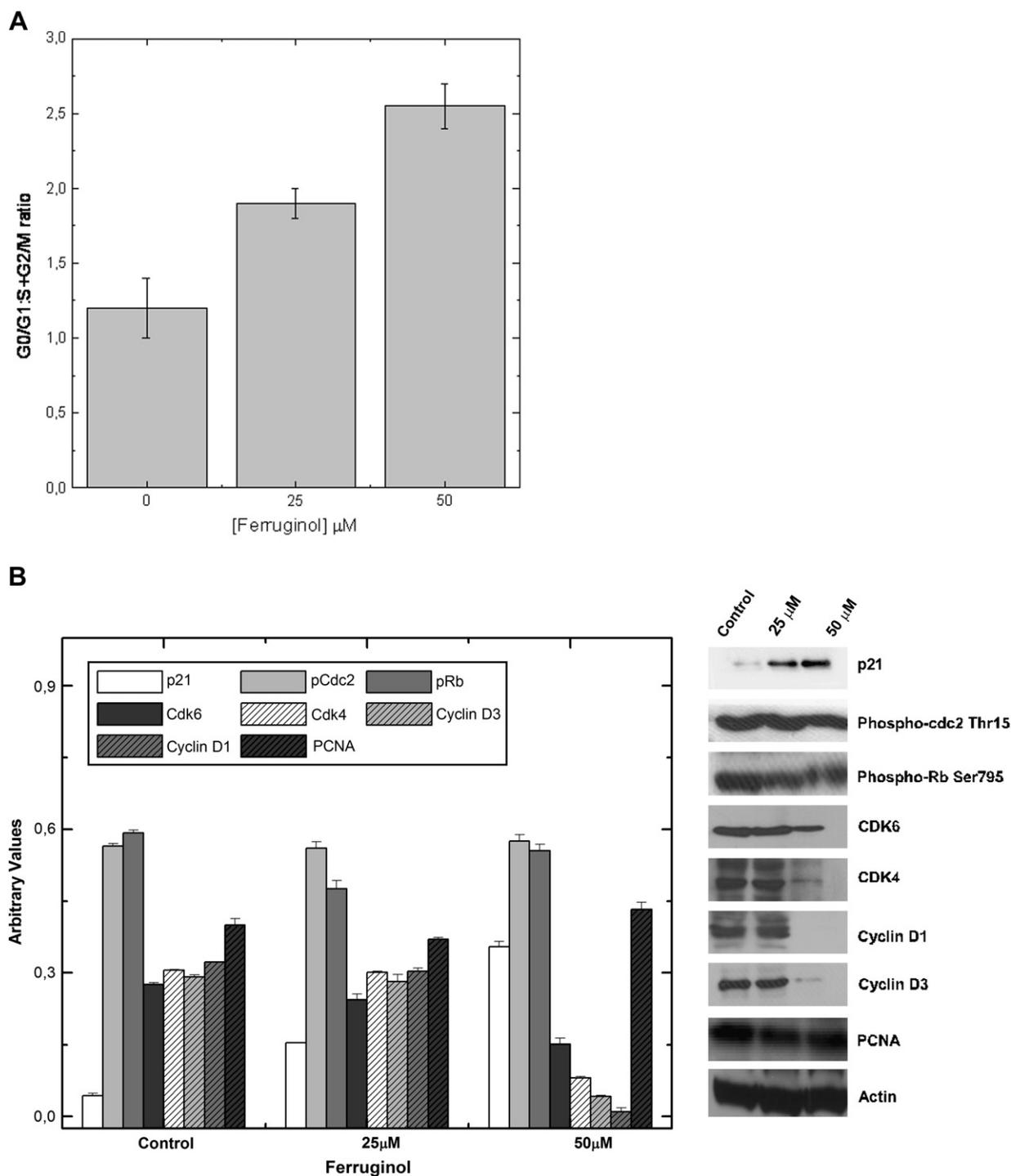


Fig. 4. Ferruginol impedes prostate cancer cell proliferation by targeting key cell cycle mediators. After treating PC3 cells with ferruginol for 24 h, cells were harvested, stained with PI and analyzed by flow cytometry (A) or lysed for Western blotting analysis (B). The number of cells in each phase of the profile and ratios of cells in resting phase (G0/G1) versus those undergoing mitosis (S, G2/M) was determined. The expression or phosphorylation of the proteins was determined by Western blot. Equal loading was confirmed by probing them for β-actin.

and because of the increasing life expectancy, its incidence is expected to further rise in the years to come [25]. Chemoprevention and intervention strategies using anticancer agents are considered as promising therapeutic options. The search for new chemopreventive and/or chemotherapeutic agents that are more effective without toxic side-effects has generated

great interest in phytochemicals with potential activity in this respect [26]. Suppression of tumorigenesis often involves modulation of signal transduction pathways, leading to alterations in gene expression, cell cycle progression or apoptosis. Apoptosis is considered as an ideal way for destroying damaged cells and also a potential target for chemopreventive

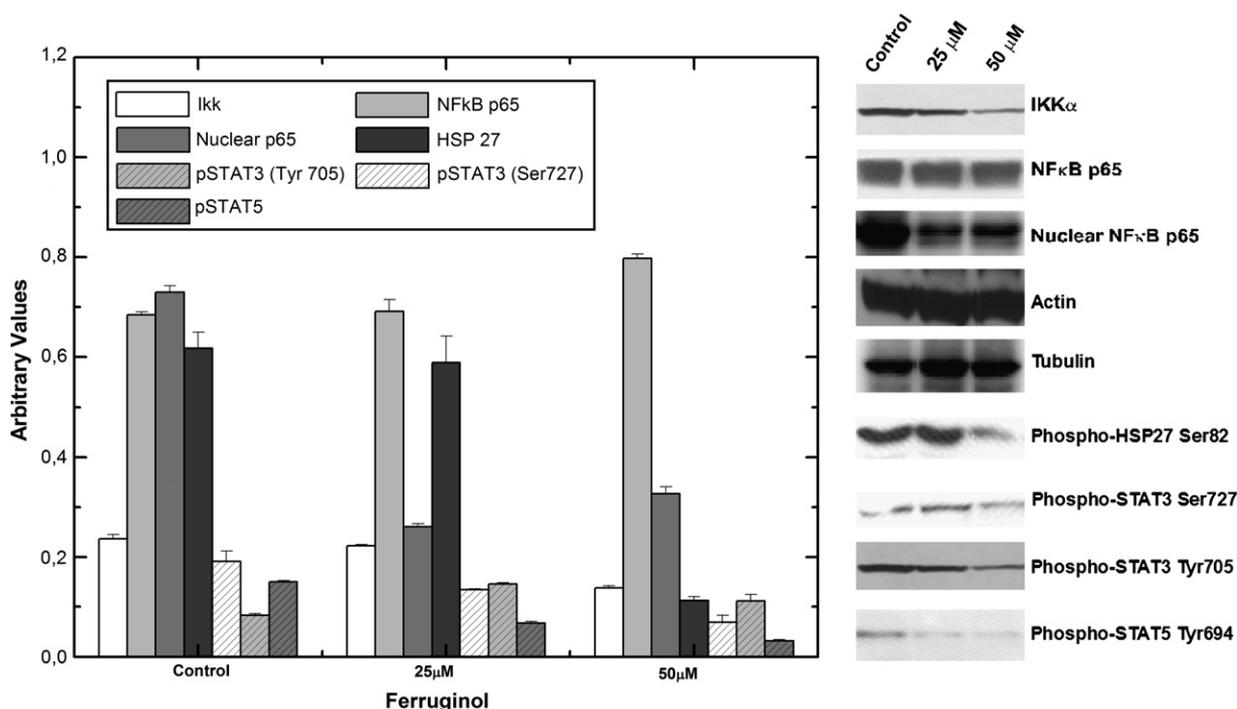


Fig. 5. Ferruginol modulates molecules that play a crucial role in the prostate cancer aggressiveness. Cells were treated with specified concentrations of ferruginol for 24 h, harvested and total cell lysates were prepared. The expression of IκB kinase, cytosolic and nuclear NFκB, and phosphorylation status of Hsp27 and STAT 3 and 5 were determined by Western blot analysis. Equal loading was probed with actin antibody to ensure equal loading.

elimination of cancer cells [27] and as a consequence targeting signaling elements controlling apoptosis may open novel therapeutic avenues [28,29]. Several plant-derived bioactive agents may have such action, at least as judged from model systems [4,9,30–33]; the present study may add ferruginol to this growing list.

Our results suggest that ferruginol is a negative regulator of cancer cell proliferation. Androgen-independent human prostate cancer cells (PC3 cells) a model that exhibits extreme therapy resistance exhibited, upon treatment with this phyto-compound, remarkable induction of apoptosis via extrinsic

and intrinsic pathways, as demonstrated by the observation of overexpression of TNFR1 and activation of caspases 8, 9 and 3. The extrinsic pathway for cell death involves plasma membrane death receptors [34]. These receptors trimerize and recruit the adaptor molecule FADD which, in turn, activates caspase 8 and also leads to the activation of downstream execution caspases [35–38]. In both pathways, activation of effector caspases leads to a series of morphological changes that are characteristic for apoptosis [28].

Ferruginol-induced apoptosis and cell growth inhibition were also accompanied by an increase of apoptosis-inducing

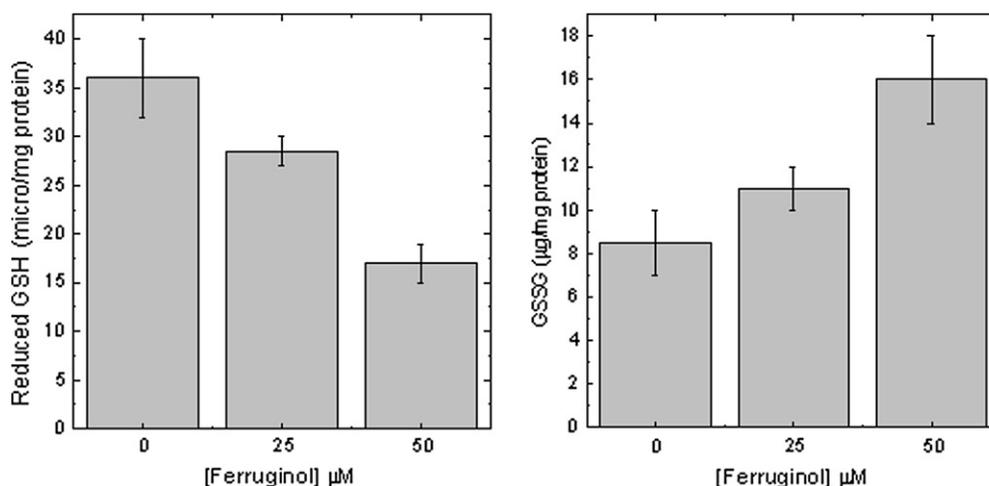


Fig. 6. Influence of ferruginol on the level of intracellular GSH and GSSG. Cells were treated with ferruginol for 24 h and the concentration of GSH and GSSG determined as described in Section 2.

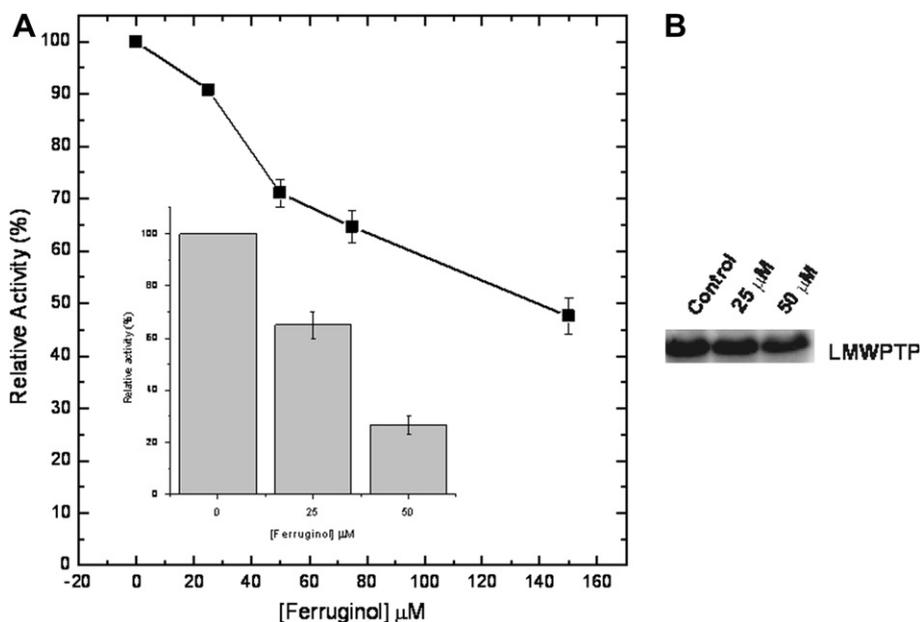


Fig. 7. Ferruginol affects low molecular weight protein phosphatase activity and expression. (A) The effect of ferruginol was examined directly in the LMWPTP immunoprecipitated from non-treated PC3 cells and also after PC3 treatment with ferruginol (inset plot). The LMWPTP specific activity was used to determine the relative activity. (B) Expression of LMWPTP was checked by Western blotting.

factor (AIF) expression and maintenance of Bax and Bcl-2 levels. AIF was identified as a mitochondrial intermembrane space protein, which is released from mitochondria and translocated to the nucleus, in response to apoptotic stimuli, and participates in peripheral chromatin condensation and the exposure of phosphatidylserine in the outer leaf of the plasma membrane. Increasing evidence supports the notion that AIF plays an important role in caspase-independent apoptosis [39,40].

Ferruginol, even at lower concentration, caused inhibition of Ras/PI3K cascade and suppression of downstream mitogenic targets such as cyclin D1. Additionally, this diterpene also induced activation of MAPK p38. The phosphatase and tensin homologue (PTEN) gene is deleted in PC3 cells. This phosphatase is defined as a tumor suppressor, since it is the major negative modulator of AKT protein, an important mediator of cell survival. It is therefore important to identify agents that can overcome the therapeutic resistant properties of PTEN deficient tumor cells. Importantly, in accordance with cell survival diminishing, ferruginol caused cell cycle arrest. Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases [41]. Ferruginol caused an overexpression of protein p21 a member of the cyclin-dependent kinase inhibitors, and downward expression of cyclin D1, cyclin D3, CDK4 and CDK6. These data showed the involvement of p21 in ferruginol-induced G₁ phase arrest, through binding to and subsequently inhibiting the cyclin-CDK activity. The active complex of cyclin D/CDK4 targets the Rb protein for phosphorylation, allowing the release of E2F transcription factors that activate G₁/S-phase gene expression. Importantly cdc-2, a key protein responsible for the entry of the cell from G₂ to M phase, remained unchanged. Cell cycle regulation and its

modulation by various plant-derived agents are gaining widespread attention in recent years. A large number of phytochemicals has been shown to inhibit cell cycle progression of various cancer cells [42].

Ferruginol decreased the phosphorylation level of STAT3, STAT5 and Hsp27. STATs are latent cytoplasmic transcription factors consisting of seven mammalian members. They become phosphorylated on Tyr residues upon activation, a post-translational modification that is critical for dimerization, nuclear import, DNA binding, and transcriptional activation [43]. The activation of STATs is mediated by the action of an upstream Janus kinase (JAK), usually JAK1 or JAK2, showing that the JAK cascade might itself be a target for therapy in prostate cancer. Ahonen and coworkers [44] demonstrated that STAT5 is activated in a significant number of human prostate cancer specimens. Additionally, these authors also reported induction of apoptosis via caspases 9 and 3 activation dependent on inhibition of STAT5 phosphorylation. Activated STAT3 was reported in many types of malignancies, such as myeloma, head and neck cancer, breast cancer, and prostate cancer [45]. Recently, it has been demonstrated that inhibition of STAT3 in tumors impeded vascular endothelial growth factor production [46]. Data from the literature have identified Hsp27 as a modulator of STAT3-regulated apoptosis after androgen ablation. Hsp27 is a 27 kDa protein of which expression is seen to be correlated with an increase of survival in response to a wide variety of physiological and environmental insults including heat, reactive oxygen species and anticancer drugs. Indeed analysis by co-immunoprecipitation and immunofluorescence confirmed that Hsp27 is able to interact with STAT3 and that STAT3 levels correlate directly with Hsp27 levels. There are some reports in the literature demonstrating that the prostate cancer Hsp27 level increases

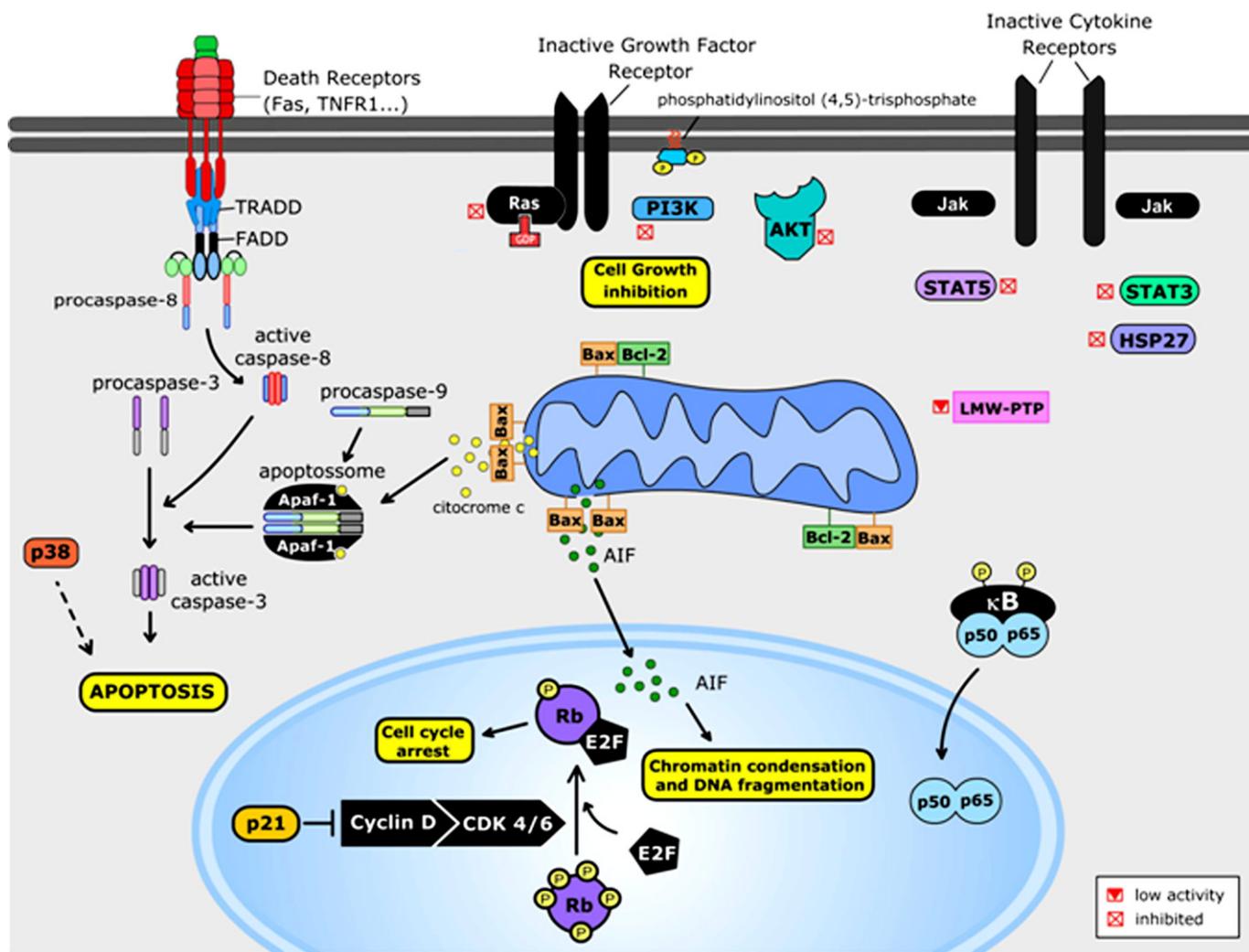


Fig. 8. Schematic representation of the molecular mechanism of ferruginol-induced PC3 cell death. Data presented in this report revealed that ferruginol exhibits multi-activities, which culminate with apoptosis induction of prostate cancer. Ferruginol caused inhibition of two important signaling cascade pathways involved in the cell survival/proliferation (Ras/PI3K and Jak/STAT). Additionally treated prostate cancer cells displayed a decrease in the phosphorylated form of Hsp27. Ferruginol-induced apoptosis was accompanied by activation of caspase 3, an increase of AIF expression and maintenance of Bax and Bcl-2 levels. AIF is released from mitochondria and translocated to the nucleus, and participates in peripheral chromatin condensation. In accordance with cell survival diminishing, ferruginol caused cell cycle arrest. Ferruginol caused an overexpression of protein p21 (a member of the cyclin-dependent kinase inhibitors), and downward expression of cyclin D1, cyclin D3, CDK4 and CDK6, which indicates cell cycle arrest at G_0/G_1 . Importantly, LMWPTP was directly and indirectly modulated by ferruginol, which indicates that this enzyme can be a target for this natural compound.

after androgen ablation and that this protein is highly expressed in androgen-independent tumors, and inhibition of Hsp27 in prostate cancer cells can increase the number of apoptotic cells (G_0-G_1), an event that seems to be associated with the decrease in the STAT3 levels [47]. These findings indicate that the anti-apoptotic effects of Hsp27 are associated with its ability to interact and stabilize the STAT3 molecule, leading to more resistant prostate cancer cells. In accordance with this notion, our results show a decrease in the phosphorylated forms of Hsp27 and STAT3, when PC3 cells were treated with ferruginol at the concentration of 50 μ M, suggesting that the pro-apoptotic and anti-proliferative actions of ferruginol might be associated with diminished function of STAT3 through the decrease of Hsp27 levels.

Another important finding is that ferruginol did not induce the NF κ B translocation to the nucleus; this result is in

agreement with the findings of Rodriguez and coworkers [17] related to the anti-inflammatory effect of ferruginol.

Recently, Chaiswing and collaborators [23] reported in a very well designed paper the effect of cellular redox state on prostate cancer cell growth *in vitro*. These authors demonstrated that during PC3 cells growth, these cells require higher ratio of reduced glutathione (GSH)/glutathione disulfide (GSSG). Based on this observation, we evaluated the redox status toward PC3 cell treatment with ferruginol. This diterpene caused a decrease of GSH and increase of GSSG, indicating a dominant effect in favor of oxidizing equivalent. Several signaling mediators can be modulated by redox modifiers, including protein tyrosine phosphatases [48,49]. To investigate the effect of ferruginol on PTPs we chose LMWPTP. The rational reason for this was based on the following aspects: (a) we have observed a high level of this phosphatase in PC3 cells

and (b) Chiarugi and collaborators [24] have reported the importance of this enzyme for cancer cell growth. Interestingly, we observed a direct effect of ferruginol on LMWPTP but also there was a correlation between cellular oxidizing equivalents and inhibition of this enzyme. The oxidation of catalytic site cysteine of PTPs, such as LMWPTP, leads to the transformation of the sulfhydrylic residue in sulfenic acid and the consequent inactivation of the enzyme due to its inability to form cysteinyl-phosphate intermediate during the first step of the catalysis [48]. Altogether, our results demonstrate that ferruginol can act as a chemical and genetic modulator of LMWPTP. These data indicate that at least in part, the anti-proliferative action of ferruginol is dependent on changing cellular redox, which is in agreement with the protective effect of GSH. This observation also confirms the importance of reducing equivalents for PC3 cell survival, as recently reported [23]. Further experiments to clarify the role of LMWPTP on prostate cancer progression are currently in progress in our laboratory.

5. Conclusion

One of the challenges of cancer therapy is to combine efficacy with few side effects and consequently improve the quality of life of the patient. Prostate cancer represents a spectrum of diseases in which the cost of cure may be substantial, with short- and long-term side effects. Therefore, new agents are needed to extend survival, improve cure rates, and avoid undesired treatment-related toxicities. In this scenario, there are at least two aims: (a) to provide therapeutic agents with a very specific target and (b) to discover agents which present differential action mechanisms in comparison with the traditional chemotherapy. In this context, ferruginol appears as an interesting bioactive compound, since it exhibits multi-activities in the signal transduction/biochemical aspects in prostate cancer cells. In summary, ferruginol negatively modulates signaling cascades, which are known to be defective in some types of prostate cancers, namely Ras/PI3K and Jak/STAT, as well as cell cycle regulators (Fig. 8). Importantly, we demonstrated for the first time that LMWPTP is directly and indirectly modulated by ferruginol, which indicates that this enzyme can be a target for this natural compound. Besides affecting signal transduction triggered by TNFR1, ferruginol also affected mitochondria permeability as demonstrated by the presence of nuclear AIF (protein involved with chromatin condensation and DNA fragmentation); however, the Bax:Bcl2 ratio remained unchanged. In general, this study provides an overview of biochemical aspects which were affected by ferruginol and in turn confirms its anti-tumor activity. This type of investigation can contribute to the development of “smart” drugs.

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