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Licochalcone-A, a novel flavonoid isolated from licorice root (*Glycyrrhiza glabra*), causes G2 and late-G1 arrests in androgen-independent PC-3 prostate cancer cells

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Abstract

Licochalcone (LA) is a novel estrogenic flavonoid isolated from PC-SPES composition herb licorice root that was reported to show significant antitumor activity in various malignant human cell lines. To better understand its anti-CaP activities, we have investigated LA-elicited growth control and induction of apoptosis using androgen-independent p53-null PC-3 prostate cancer cells. LA induced modest level of apoptosis but had more pronounced effect on cell cycle progression arresting cells in G2/M, accompanied by suppression of cyclin B1 and cdc2. It also inhibited phosphorylation of Rb, specifically phosphorylation of S780 with no change of phosphorylation status of T821, decreased expression of transcription factor E2F concurrent with reduction of cyclin D1, down-regulation of CDKs 4 and 6, but increased cyclin E expression. These findings provide mechanistic explanation for LA activity and suggest that it may be considered as a chemopreventive agent and its anticancer properties should be further explored.

Keywords: Licochalcone-A; Cell cycle control; Prostate cancer

Prostate cancer (CaP) is a leading cause of cancer deaths in American men [1]. Current therapies for CaP provide short-term tumor regression; ultimately CaP progresses to the AI state, with a median survival of 18 months [2–7]. These grim medical statistics underscore the need for development of novel strategies to better manage and control CaP, and have provided impetus to scientists and industries to actively search for, isolate, identify, and characterize agents that might be developed into alternative anti-CaP therapies. Namely, compounds that induce growth arrest, apoptosis, and differentiation, particularly androgen-independent cells, may provide alternative therapeutic approaches in cases where chemotherapy is not effective. In this context, a promising lead was found in the herbal supplement PC-SPES, as it was shown to have significant clinical efficacies for both hormone-dependent and refractory forms of the disease [8–17]. Unfortunately, some PC-SPES samples were subsequently reported to be contaminated by prescribed medications; this led to its abrupt discontinuation leaving thousands of regular users in a state of confusion and dismay [8,18–20].

Screen of bioactivities of composition herbs of PC-SPES has revealed that extracts derived from *Scutellaria baicalensis* and *Glycyrrhiza uralensis* contained biological activities comparable to PC-SPES, as evidenced by reduction in proliferation of CaP cells and lowered

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expression of two prostate specific genes, the androgen receptor AR and prostate specific antigen PSA [21]. Further analysis of extracts derived from these two herbs led to the successful isolation and identification of three bioactive chemicals, respectively, baicalin, baicalein, and licochalcone-A (LA) [22–28]. Notably, LA is an oxygenated chalcone extracted from PC-SPES composition herb licorice root, *G. uralensis* [18,22,28]. As a novel flavonoid, LA possesses multiple bioactive properties including anti-parasitic, estrogenic, and antitumor activities [22,29–38].

Glycyrrhiza root has been shown to decrease circulating levels of testosterone in men [28,39,40]. Moreover, DiPaola and co-workers [22] reported that LA isolated from *Glycyrrhiza* root also demonstrated potent antitumor properties when assayed using CaP, breast, and leukemia cells. LA also induced apoptosis in MCF-7 breast cancer and HL-60 leukemia cells, as assayed by cleavage of poly(ADP-ribose) polymerase, reduced expression of bcl-2, and diminution of the bcl-2/bax ratio [22,28]. Since these attributes raise the possibility that LA may be a potent and efficacious agent for treating various malignancies including CaP, we performed experiments to further delineate its anti-CaP properties. In the current study, we provide evidence showing that LA elicited time- and dose-dependent suppression of proliferation of hormone-independent PC-3 cells, which were correlated with down-regulation of the expression of regulatory proteins involved in control of cell cycle checkpoints. Proteins adversely affected by LA included proliferating cell nuclear antigen (PCNA), DNA polymerase δ, Rb and E2F, cyclins B1 and D1 and their catalytic partners, respectively, cdc2 and CDKs 4 and 6. Unexpectedly LA also increased the expression of cyclin E. These results are significant in that they provide a mechanistic framework for further exploring the use of LA as a novel anti-CaP agent for treating/managing CaP.

Materials and methods

Cell culture. Human PC-3 CaP cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in RPMI-1640 media (Mediatech, Pittsburgh, PA) containing L-glutamine, supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Mediatech, Pittsburgh, PA). Culture media were changed every 3–4 days and cells were split once a week, as described [41,42].

Preparation of licochalcone-A. Licochalcone was purchased from Calbiochem (La Jolla, CA) and prepared as a 5.9 mM stock in DMSO (Sigma Chemical, St. Louis, MO). Prior to use, the LA stock was further diluted in RPMI-1640 media and added to cultures to give the final indicated concentrations. Untreated cultures received the same amount of solvent (<0.2% v/v DMSO).

Effect of LA on cell growth and morphology. PC-3 cells seeded at 5×10^4/ml in T25 flasks (Sarstedt, Newton, NC) were first allowed to attach overnight and then incubated with 0, 12.5, and 25 μM LA. At various times after treatment, cells were harvested by trypsinization. Control and treated cells were stained with trypan blue and cell number and viability were determined using a hemocytometer [41,42]. To further assess the effects of LA on PC-3 proliferation, cells were fixed with acetone/methanol (50%/50%), washed with PBS, and incubated with Wright stain follow by counterstain with eosin (Sigma Chemical, St. Louis, MO). Cell morphology was then evaluated by light microscopy.

Detection of apoptosis and cell cycle analysis by flow cytometry. PC-3 cells treated with different concentrations of LA (0, 12.5, and 25 μM) for 1–3 days were harvested, washed with PBS, and then stained with 1.0 μg/ml of 4,6-diamidion-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) in a solution containing 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO) at pH 6.8, as previously described [41,43]. Cell cycle phase distribution and the percentage of apoptotic cells were determined using a FACScan flow cytometer and the CellFit software [44,45]

Protein extraction and Western blot analysis. Control and treated cells were rinsed with ice-cold PBS, suspended in buffer (50 μl/10^6 cells) containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5% NP40, and 5% glycerol supplemented with 0.5 mM PMSF, 10 μg/ml each of aprotinin, pepstatin, leupeptin, and lysed by 3 freeze/thaw cycles [27,46]. The extracts were centrifuged and the clear supernatants were stored in aliquots at −70 °C. Protein concentrations were measured with Pierce protein assay reagent (Pierce Chem., Rockford, IL). For Western blot analysis, 10 μg proteins were boiled for 5 min in Laemmli buffer and separated on 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were then transferred to nitrocellulose membranes (Schleicher and Schuell Biosciences, Keene, NH) by a semi-dry transfer method. After blocking with TBST containing 5% low-fat milk, the membranes were probed for the level of expression of cyclin B1, D1, E, cdc2, CDK 2,4,6, Rb, PCNA, and ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Site specific phosphorylated Rb antibodies (Ser 780 and Thr 821) were purchased from Biosource International (Camarillo, CA). Antibody to DNA polymerase δ (78F5) was used as previously described [47]. All antibodies used in the experiments were diluted at 1:1000. Specific immunoreactivity was demonstrated by enhanced chemiluminescence (ECL) or color reaction using procedures detailed in the manufacturer's protocol (Kirkegared and Perry Laboratories, Gaithersburg, MD).

Statistical analysis. Significance in the various cellular and biochemical parameters between control and LA treated samples was determined using the Student t test. The results are presented as means ± SD.

Results

Inhibition of cell proliferation and induction of cell cycle arrest in G_2/M following LA treatment of PC-3 cells

Although DiPaola and co-workers [22] have demonstrated that LA exerted anti-proliferative effects against several malignant cell types, its growth inhibitory properties in CaP cells remained largely unknown. To gain additional insights into LA-elicited suppression of proliferation, PC-3 cells were treated with different concentrations (0, 12.5, and 25 μM) of LA for varying time periods (1–3 days). The anti-proliferative effects of LA at either concentration were not apparent on day 1 ($p = 0.4423$, 0.1805), but clearly became evident after 2–3 days of exposure, showing an approximately

65–80% growth inhibition on day 2 ($p = 0.0472$, 0.0230) and 55–83% growth reduction on day 3 ($p = 0.0242$, 0.0015) (Fig. 1A). This effect did not appear to be cytotoxic, since cell viability was greater than 90%, as determined by trypan blue exclusion (data not shown). The growth suppression activity of LA correlated with overall changes in the expression of DNA polymerase δ and its associated protein, proliferating cell nuclear antigen (PCNA). A 20% reduction in DNA polymerase δ (assayed by immunoreactivity against the 78F5 antibody) occurred on day 1, which became much more pronounced by day 3 (~80% suppression). Correspondingly, a more modest decrease (5–30% inhibition) in the expression of PCNA was also observed (Fig. 1B). Other than the clearly visible cytostatic effect, LA induced little morphological changes in these cells. The significantly reduced cell number in cultures was not accompanied by detachment from the flask, rounding up of the cells, and condensation of cytoplasm and nuclei, the features typical of apoptosis, even when challenged with 25 μM LA (Fig. 1C).

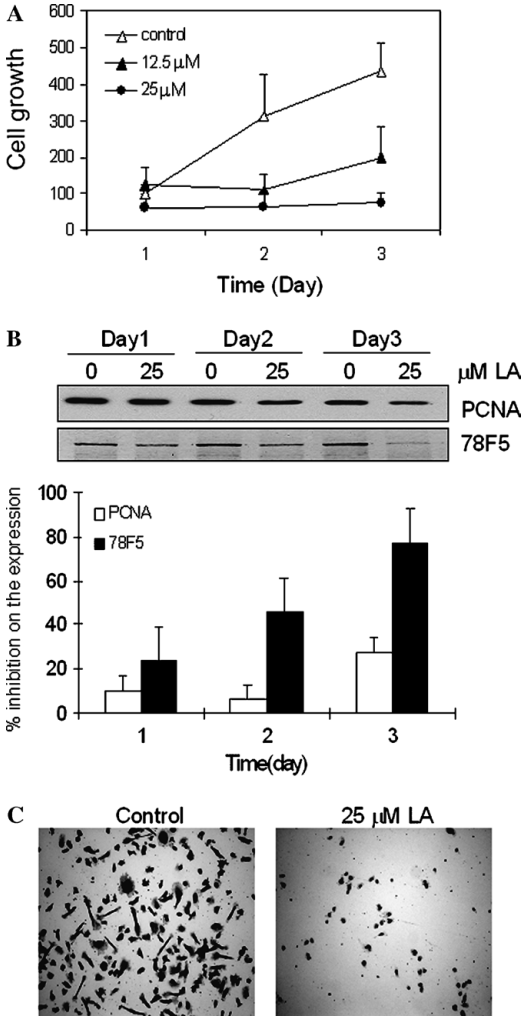

Fig. 1. Effect of LA on proliferation of androgen-independent PC-3 cells. (A) Cells were treated for 1–3 days with 0, 12.5, and 25 μM LA. Growth was measured by counting the cell number using a hemacytometer. Results were averaged from three independent experiments and presented as means ± SD. (B) Relative changes in expression of PCNA and DNA polymerase δ (measured by immunoreactivity to monoclonal antibody 78F5) in control and LA treated PC-3 cells. Western blots were used to probe the expression of PCNA and 78F5 in day 1–3 LA-treated cells (0 and 25 μM). The intensities of the immunoreactive bands were separately quantified by densitometry, with the expression of actin serving as a control to verify equivalent protein loading. The data were presented in the bar graphs as percentage inhibition, with control values for each day of treatment showing as 100%. (C) Cell morphology in control and 25 μM LA-treated PC-3 cells (magnification 100×).

The dose-dependent effect on cell cycle parameters of PC-3 cells after exposure to LA was investigated by flow cytometry, since earlier studies have demonstrated that a 72 h exposure to LA reduced the viability of PC-3 cells [22]. Cells incubated with different concentrations of LA (0, 12.5, and 25 μM) for 1–3 days were examined for their distribution in the G_1, S, and G_2/M phases of the cell cycle. Fig. 2 shows that cell treatments with 25 μM LA resulted in an increase in the percentage of cells in the G_2/M phase at all time points (1–3 days) whereas in cells treated with 12.5 μM LA, G_2/M block was observed on day 3 only (Fig. 2). Similarly, the effects of LA on the induction of apoptosis were also investigated by flow cytometry. The amount of apoptotic cells was measured based on the appearance of a "sub-G1" peak. Although time- and concentration-dependent increase of apoptosis was observed in LA-treated cells, the magnitude of change was relatively small in comparison to the observed decrease in cell proliferation. A 3-day treatment with 25 μM LA only elicited a 5–6% additional increase in apoptotic cells (data not shown). These findings indicate that at these LA concentrations growth inhibitions in PC-3 cells can be primarily attributed to induction of G_2/M block, with only minor contribution from the induction of apoptosis.

Effect of LA on the expression of cell cycle regulatory proteins in PC-3 cells

To further elucidate mechanisms of induction of G_2/M arrest by LA, changes in protein kinase complex (cyclin B1/cdc 2) associated with the control of transition of this phase of the cell cycle were investigated in cells treated with 25 μM concentration of LA. Western blot analysis revealed a strikingly similar degree of inhibition in the level of cyclin B1 at all time points (1–3 days); in contrast, reduction in its catalytic partner-cdc2 was much more time-dependent and did not become evident until days 2–3 after LA treatment (Fig. 3A).

The effects of LA on the expression of other cyclins and their corresponding protein partners were also investigated. These included cyclins D1 and E and CDKs 2, 4, and 6. Exposure of cells to 25 μM LA resulted in a time-dependent reduction in the expression

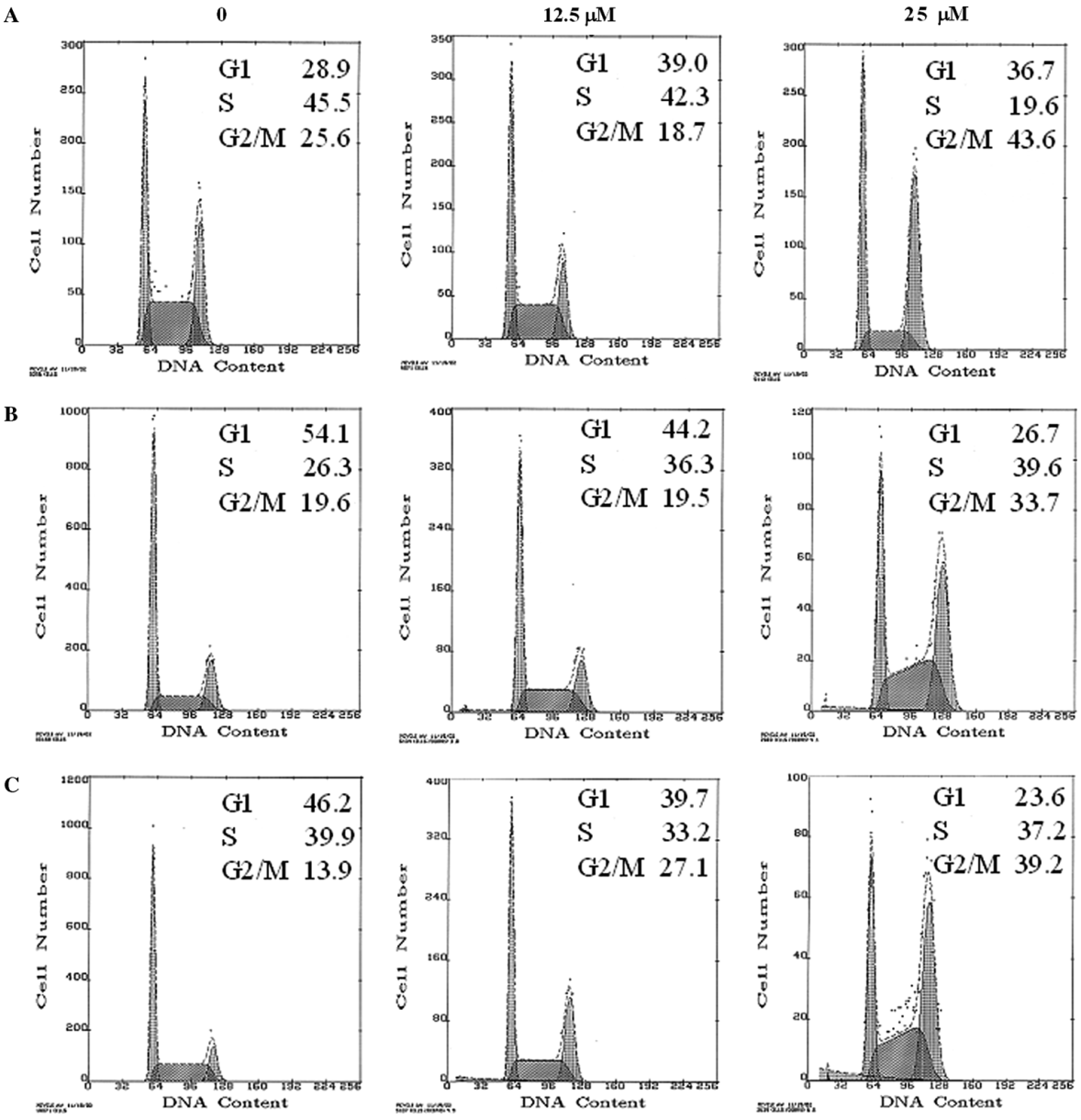

Fig. 2. Cellular DNA content frequency histograms showing the cell cycle phase distribution and apoptosis of PC-3 cells following 1–3 day (A–C) treatment with different concentrations of LA. Flow cytometric analysis was performed as described in Materials and methods. Note distinct accumulation of cells in G_2/M and modest proportion of cells with fractional DNA content (sub-G_1 cell population; typical of apoptosis) in cultures treated with LA.

of cyclin D1 accompanied by down-regulation of CDK 4 and a delayed decrease in CDK 6 on day 3 (Fig. 3B). Curiously, a time-dependent increase in cyclin E without commensurate change in CDK 2 was also observed (Fig. 3C). These contrasting changes in cyclins D1 and E by LA are in concordance with time-dependent transition of the cell population from G_1 to S, as revealed by flow cytometry analysis (Fig. 2).

The observed changes in these cyclins and cyclin-dependent kinases might subsequently affect the state of phosphorylation of retinoblastoma (Rb). The phosphorylation pattern of Rb is an established indicator of the proliferative state of the cell. Hypophosphorylated Rb is found in the noncycling G_0 and early G_1 cells, whereas hyperphosphorylated Rb is characteristic of proliferating cells in the S- and G_2/M phases. The status of Rb phosphorylation controls the availability of transcription factor E2F responsible for growth control. Therefore, the effects of LA on Rb and E2F were further investigated. Western blot analysis demonstrates that 25 μM LA caused a significant decrease in the expression of Rb (>60% inhibition), particularly the hyperphosphorylated form of Rb (Fig. 4A). Since cyclin D1/CDK 4 modulates Rb phosphorylation at S780 both in vivo

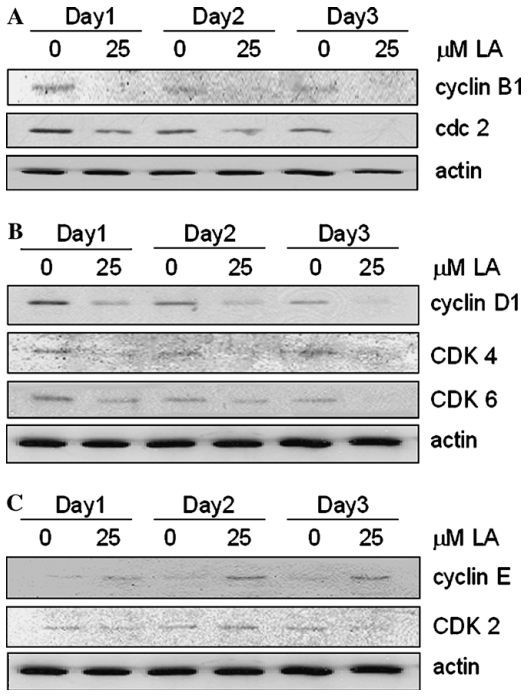

Fig. 3. Changes in cell cycle regulatory proteins in control and 1–3 day 25 μM LA-treated PC-3 cells. Expression of cyclin B1, cdc2, and actin (A), cyclin D1, CDK4, CDK6, and actin (B), and cyclin E, cdk2, and actin (C), respectively, was determined by immunoblot analysis as detailed in Materials and methods.

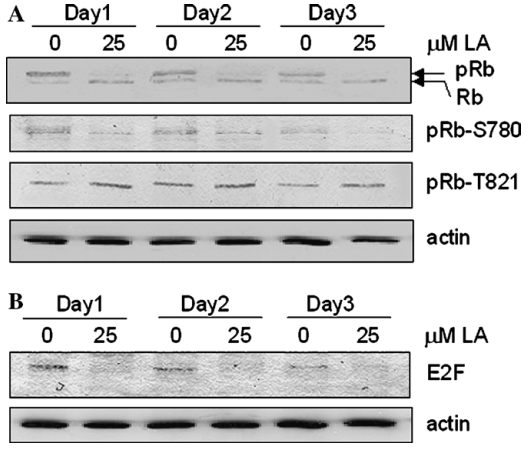

Fig. 4. Effect of LA (25 μM) on expression of Rb and E2F in PC-3 cells. (A) Time-dependent changes in the profile of Rb, pRb (S780), pRb (T821), and actin in control and treated cells. (B) The expression of E2F and actin following treatment by LA (25 μM).

and in vitro [48] whereas cyclin E/cdk2 affects Rb phosphorylation at T821, phosphorylation of these two specific amino acid residues was monitored by immunoblot analysis using phosphorylation site-specific antibodies. Following LA treatment a significant reduction was found at S780 and not T821 (Fig. 4A), which is consistent with the observed effects of LA on cyclin D1/CDK 4 expression (Figs. 3B and 4A). In addition to the changes in Rb phosphorylation, down-regulation of E2F by LA was also observed; this change may additionally contribute to the observed growth suppression (Fig. 4B). Taken together, these findings suggested that LA exerts multiple effects in cell cycle progression resulting in the specific cell cycle checkpoint arrest of PC-3 cells.

Discussion

Licorice root is one of several commonly used herbs already identified by the National Cancer Institute as containing chemopreventive attributes [49,50] Several isoflavons belonging to a subclass of the flavonoid compounds, as primarily represented by glabridin and shown to possess a plethora of biological activities, have been isolated from licorice root. A number of triterpenoids, exemplified by glycyrrhizin and glycyrrhetinic acid, have also been identified from this plant. Glycyrrhizin and glycyrrhetinic acid are best known for their sweetness, being ~170 times as sweet as sucrose [50]. In addition, they also have been reported to possess potent bioactivities. These range from hydrocortisone-like anti-inflammatory effects to amelioration of ischemia–reperfusion injury and improvement of renal functions [50]. Numerous polyphenolics have been isolated from the dried root of *Glycyrrhiza* species. These include phenolic acids, flavones, chalcones, and isoflavonoids. Licorice polyphenols may be responsible for the antioxidant activity of licorice. Licochalcones B and D strongly inhibited superoxide anion production in the xanthine/xanthine oxidase system. In previous studies by DiPaola and co-workers, LA has been shown to exert potent antiproliferative effect in a variety of tumors, and also induced apoptosis in MCF-7 and HL-60 cells [8,22].

No effective treatment currently exists for androgen-independent, metastatic CaP. This form of the disease is known to be radiation and chemo-resistant, with extremely poor prognosis and an average survival of only 18 months. As a prelude to the development of alternative therapies using common herbs with medicinal properties, we evaluated in the present studies the antineoplastic effects of LA, especially focusing its influence on cell cycle events. In accordance with the previous findings of DiPaola and co-workers [22], our data showed that LA effectively inhibited proliferation of PC-3 cells. Whereas published studies primarily focused on the apoptogenic properties of LA using MCF-7 and HL-60 cells, results of this communication show that in PC-3 cells LA induced multiple effects on the cell cycle progression regulatory machinery that led to the induction of G_2/M checkpoint arrest. Conceivably also, LA may induce very late G1 (and may be S) arrest, akin to the notion of hypermitogenic arrest, a concept previously advanced by Blagosklonny [51].

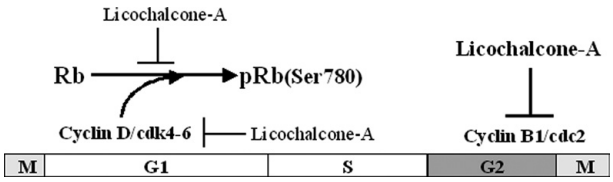

Fig. 5. Proposed mechanism of action in cell cycle control by LA in PC-3 cells. The scheme depicts that the anti-proliferative property of LA is mechanistically linked to multiple changes in the expression of cell cycle regulatory proteins. Most profound suppression occurred in cyclin B1 expression and phosphorylation at S780 of the Rb, suggesting that these may represent the primary targets of LA.

These effects were largely mediated through changes in abundance and activity of different cyclin/CDK complexes, reduction in Rb expression, and in its phosphorylation at specific amino acid residues (Fig. 5). Notably, the observed down-regulation of cyclin B1/cdc2 by LA may be mechanistically related to LA-induced G_2/M block and growth inhibition in PC-3 cells [22]. Moreover, we also showed that LA failed to curtail cell progression from G_1 to S phase even with a significant reduction in the expression of Rb/pRb (S780) and E2F. A plausible explanation for these observations might relate to the p53 null status of PC-3 cells, making enforcement in G_1/S transition ineffective relative to other cell cycle checkpoints. It is also noteworthy that upregulation of cyclin E expression by LA did not have the anticipated change in the status of phosphorylation of pRb at T821, which might be expected to impair the binding of Rb to E2F and hence attenuate cell cycle phase transition between G_1 and S.

The exact mechanism by which LA induces the down-regulation of cyclins and other cell cycle regulatory proteins remains to be investigated. Conceivably LA could promote degradation of cyclins, in a manner analogous to that recently reported for retinoic acid, fenretinide, and 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid [52]. This induced turnover of cyclins could be mediated by the proteasomes, or alternatively, secondary to increased expression of UBE1L (ubiquitin-activating E1-like) which has recently been reported to be under the control of retinoic acid in promyelocytic leukemia cells [53,54] In this context, LA might bind specific cellular targeting proteins that directly or indirectly lead to the observed degradation of cyclins. We have explored this possibility by covalently attaching LA to epoxy-activated agarose beads to form an LA-immobilized affinity platform for isolating and identifying proteins that bind with specificity and affinity with LA. This approach has resulted in the isolation of a number of bound proteins (data not shown). Currently we are refining experimental conditions to better define and characterize their properties. It is noteworthy that other chemicals isolated from *Glycyrrhiza* species, specifically glycyrrhizin and glabridin, have been reported to bind a wide range of proteins, many with important cellular functions. Examples include angiogenin-1, an angiogenesis inducer with RNase activity, casein kinase II, type IIA phospholipase A_2, high mobility group proteins 1 and 2, and the estrogen receptor [55–61].

In summary, the foregoing results provide useful information on the mechanism of action of this novel flavonoid, in the context of its demonstrated antiproliferative effects in androgen-independent PC-3 cells. These data provide further support for the notion that *G. uralensis* and its active component LA should be further explored as a possible chemopreventive modality as well in terms of their possible effectiveness in treatment of prostate cancer.

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