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Deguelin inhibits the growth of colon cancer cells through the induction of apoptosis and cell cycle arrest

G. Murillo[a], G. I. Salti[a], J. W. Kosmeder II[b], J. M. Pezzuto[b], R. G. Mehta[a,*]

[a]*Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, IL 60613, USA*
[b]*Program of Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, IL 60612, USA*

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Abstract

As previously demonstrated, deguelin [(7a*S*, Ba*S*)-13, 13a-dihydro-9,10-dimethoxy-3,3-dimethyl-3*H*-bis[1]benzo-pyrano[3,4-b: 6′,5′-*e*]pyran-7(7a*H*)-one mediates anti-proliferative properties in a variety of cell types. In the present study, deguelin was found to suppress the growth of HT-29 colon cancer cells with an IC_{50} of 4.32×10^{-8} M. The cells were arrested in the G1-S-phase of the cycle. Investigations of G1/S regulatory proteins by Western blot analyses showed an upregulation of p27, and decreased expression levels of cyclin E and CDK4. Furthermore, by 24 h, exposure to deguelin resulted in an increase in the hypophosphorylated form of Rb. Since hypophosphorylated pRb binds to and inactivates E2F1, additional studies were performed and downregulation of E2F1 was observed after 24 h of treatment with deguelin. These results are consistent with the observation that deguelin arrested cells in the G1-S- phase. In addition, based on ethidium bromide/acridine orange staining, detection of digoxigenin-labelled genomic 3′-OH DNA ends, and DNA laddering, it was found that deguelin exerts its growth inhibitory effects via the induction of apoptosis. Based on these data, the potential of deguelin to serve as a cancer chemotherapeutic agent for colon cancer may be suggested.

Keywords: Deguelin; Cell cycle; Apoptosis; Colon cancer; HT-29; G1 arrest

1. Introduction

During the past 20 years, the identification of novel cancer preventive agents has received considerable attention [1–3]. The mechanism of action of a chemopreventive agent is often related to the stage of carcinogenesis at which it is active [4]. For example, antioxidants are often anti-initiators, whereas inhibitors of ornithine decarboxylase (ODC), agents that obstruct cell differentiation or induce programmed cell death, are generally anti-promoters. Chemotherapeutic agents, on the other hand, are used for established tumours that are aggressively progressing. The major difference between chemopreventive and chemotherapeutic agents is that chemopreventive agents are generally non-toxic, whereas chemotherapeutic agents are highly cytotoxic with narrow therapeutic ranges. Thus, there appears to be an ill-defined discrimination between the chemopreventive and chemotherapeutic effects of a compound, especially if the compound is active during the later stages of carcinogenesis. Under these circumstances, chemopreventive agents acting during the progression stage of carcinogenesis can be considered as potential chemotherapeutic agents. Since chemopreventive agents, by definition, must be non-toxic, these agents may serve as safer alternatives. Accordingly, a variety of chemopreventive agents have been considered as potential adjuvant chemotherapeutic agents [5]. These include retinoids, cyclooxygenase (COX) inhibitors, ODC inhibitors, flavonoids, triterpenes, and others [6].

Deguelin, a rotenoid, was identified as a potential chemopreventive agent from *Mundulea sericea* within the Leguminosae family (Fig. 1). This was accomplished during the process of activity-guided fractionation in an effort to identify novel chemopreventive agents [7]. Deguelin is a potent inhibitor of ODC activity with an IC_{50} of 0.7 ng/ml in cultured mouse 308 cells, and has

* Corresponding author. Tel. +1-312-413-1156; fax: 1-312-996-9365.
E-mail address: raju@uic.edu (R.G. Mehta).

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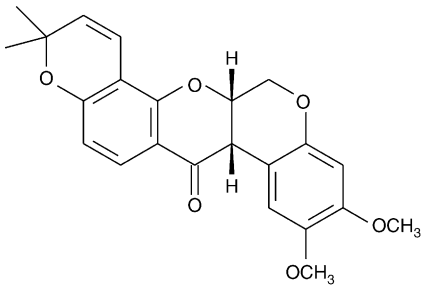

Fig. 1. Chemical structure of deguelin.

been shown to inhibit phorbol-12-myristate 13-acetate (TPA)-induced ODC activity in mouse skin epidermis [8]. Typically, rotenoids are toxic piscicidal agents, however, deguelin is tolerated by mammals at concentrations of up to 4 mg/kg body weight without inducing toxic effects when administered intragastrically to rats [9]. Unlike rotenone, deguelin does not inhibit microtubule assembly or tubulin polymerisation [10]. Due to the activity mediated by deguelin in in vitro models, it has been evaluated as a potential chemopreventive agent. For example, deguelin inhibited the development of carcinogen-induced precancerous lesions in mouse mammary gland organ cultures (MMOC), and suppressed the development of skin papillomas in a two-stage mouse skin carcinogenesis model [11]. In addition, deguelin inhibited tumour multiplicity in a rat mammary carcinogenesis model [11]. Although studies have provided suggestive evidence for the chemoprotective and therapeutic roles of deguelin in different cell types, the mechanism of action by which this rotenoid functions has not been described.

Each year, approximately 140,000 new cases of colorectal cancer are diagnosed in the United States, making it the third leading cause of cancer deaths [12]. Cytotoxic chemotherapy has been used to treat patients with advanced colorectal cancer with limited success, often at the expense of severe side-effects. Therefore, identification of novel chemotherapeutic agents for colon cancer with fewer side-effects is warranted.

2. Materials and methods

2.1. Reagents and chemicals

Roswell Park Memorial Institute (RPMI) 1640 was purchased from Life Technologies, Inc. (Rockville, MD). Monoclonal antibodies against proliferating cell nuclear antigen (PCNA; clone PC10), E2F-1 (clone KH129), cyclin E (clone HE12) were purchased from NeoMarkers (Fremont, CA). Mouse monoclonal anti-p27 antibody (clone 57) was obtained from Transduction Laboratories (Lexington, KY). Goat polyclonal anti-actin antibody (clone I-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Mouse monoclonal anti-CDK2 (clone G120-72), rat monoclonal anti-CDK4 (clone ACD1) and mouse polyclonal anti-RB antibodies (clone G3-245) were purchased from BD Pharmingen (San Diego, CA). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Deguelin was synthesised from commercially available rotenone (Aldrich, Milwaukee, WI) using a four-step process [13]. The purity of the final product was 97–98%. Deguelin was dissolved in 100% ethanol for all of the *in vitro* studies.

2.2. Cell lines and culture conditions

HT-29, SW620 and SW-480 colon cancer cell lines were obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotic-antimycotic solution (10 units/μl penicillin, 10 μg/μl streptomycin and 25 μg/ml amphotericin B) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell growth and toxicity

For determination of proliferation, HT-29 cells were seeded at a density of 2×10^4 per well onto 12-well cell culture plates and allowed to adhere for 24 h. After incubation with or without deguelin, cells were detached with trypsin and the cell number was determined by the Coulter counter. In order to determine whether the effects of deguelin were toxic to the cells, a recovery experiment was carried out. Deguelin was removed from the media after day five, fresh medium was added and the cells were incubated for an additional 48 h prior to being evaluated for cell proliferation.

2.4. Cell cycle analysis

HT29 cells were treated without or with 1.0 μM deguelin for 6, 18, 24, 48 or 72 h, and then harvested with trypsin and washed with phosphate-buffered solution (PBS). After the final wash, the cells were resuspended in 1.0 ml of PBS, 9 ml of ice cold 70% ethanol were added, and the samples were stored at −20 °C until staining. In preparation for staining, cells were washed three-times with PBS and resuspended in 0.3 ml of citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 0.05% v/v dimethylsulphoxide (DMSO), pH 7.6). The samples were then stained with propidium iodide using the detergent-trypsin method described by Vindelov and colleagues in Ref. [14].

2.5. Assessment of apoptosis

Induction of apoptosis was determined by acridine/ethidium staining [15]. Cultured cells were centrifuged

and suspended in PBS and the mixture of dyes was added. Fluorescent microscopy was used to identify non-viable cells with nuclei stained bright orange. Viable cells exclude ethidium bromide and stain bright green. A quantitative assessment was made by determining the percentage of apoptotic cells, whose nuclei were highly condensed or fragmented.

Next, an ApopTag Peroxidase *in situ* Apoptosis detection kit purchased from Oncor (Gaithersburg, MD) was used to detect directly digoxigenin-labelled genomic 3′-OH DNA ends generated during apoptosis. Apoptosis was also evaluated by examining the characteristic pattern of DNA laddering generated in the apoptotic cells using gel electrophoresis. Briefly, after 72 h of treatment with vehicle (ethanol) or deguelin dissolved in ethanol, cells were suspended in PBS, and lysed in 0.5 ml of lysis buffer (0.6% sodium dodecyl sulphate (SDS) + 10 mM ethylene diamine tetra acetic acid (EDTA)). NaCl was added to a concentration of 1 M and mixed by inversion. The mixture was left at 4 °C for at least 12 h and then centrifuged. RNase A was added to the supernatant, incubated at 37 °C for 30 min, and extracted with phenol/chloroform. The samples were electrophoresed through a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

2.6. Western blot analyses

Treated and untreated cells were lysed in freshly prepared extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 0.1% Nonidet (N)P-40, 10% glycerol, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl flouride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 45 min on ice. Lysates were centrifuged, the supernatant was collected, and protein concentration was determined using a modified Lowry method (Bio Rad, Hercules, CA).

Samples were separated (200 μg of protein per lane) on 7.5–10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk, then incubated with primary antibody for 2 h at room temperature. PCNA, Actin and Cyclin E were prepared as 1 μg/ml whereas Rb, CDK2, and E2F1 were diluted as 2 μg/ml. The p27 antibody was used at a concentration of 0.5 μg/ml. The secondary anti-mouse antibody was incubated with the membrane for 45 min at room temperature. The chemiluminescence reaction was performed using the ECL system from Amersham Pharmacia Biotech (Piscataway, NJ) according to their protocol. Bands were analysed by densitometry using Kodak Digital Camera 2D software. The band intensities from the proteins of interest were compared with those of actin and the relative intensity ratios were calculated. Western blot analyses of the proteins of interest were examined in three separate experiments.

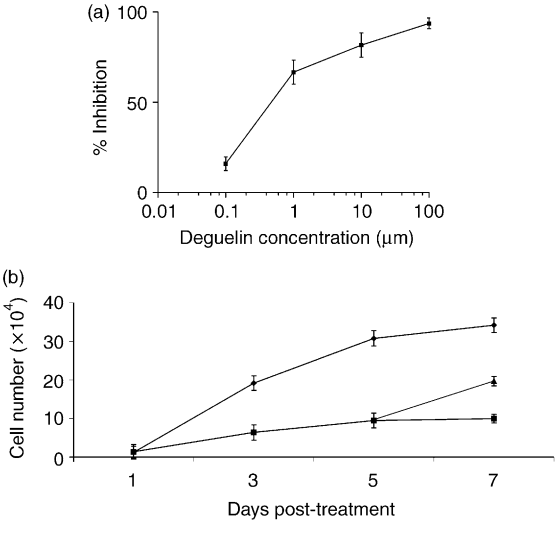

Fig. 2. Effects of deguelin on HT-29 cell proliferation. [a] Deguelin dose response curve. [b] Time course. Vehicle only (●), 1 μM deguelin for 7 days (■), 1 μM deguelin for 5 days, followed by vehicle for 2 days (▲).

3. Results

3.1. Growth inhibition in HT-29 cells

Effects of various concentrations of deguelin (10 nM–10 μM) were examined in HT-29 colon cancer cells. Deguelin mediated growth inhibition in a dose-dependent manner (Fig. 2), with 70% inhibition at a concentration of 1 μM and an IC_{50} of 4.32×10^{-8} M. The maximum anti-proliferative response of deguelin was observed after 5 days of treatment. Anti-proliferative effects were also evident in the colon cancer cell lines SW-620 and SW-480 with an IC_{50} of 4.62×10^{-7} M and 4.59×10^{-8} M, respectively (data not shown). A recovery experiment was performed to determine if the effect of deguelin on the HT-29 cells was reversible. Results showed that when media containing deguelin was replaced by fresh media, the cells were able to recover and continue to grow, thus suggesting that deguelin functions by a cytostatic mechanism and continuous exposure is needed to maintain and achieve its anti-proliferative effect (Fig. 2b).

3.2. Cell cycle analysis

In order to determine the phase of the cell cycle at which deguelin exerts its growth inhibitory effect, HT-29 cells were treated with deguelin for 6–72 h and analysed by flow cytometry. The effects of deguelin (1 μM) on cell cycle progression are shown in Fig. 3. An arrest in the G1 phase was apparent at 24 h and remained evident after 72 h of treatment. In addition, treatment with

deguelin for 72 h was associated with a sub-G1 peak (Fig. 3), suggesting that deguelin may be inducing apoptosis in these cells. Similarly, deguelin was found to cause a sub-G1 peak in SW-620 and SW-480 cells (data not shown).

3.3. Induction of apoptosis

To further confirm the induction of apoptosis as a result of treatment with deguelin, treated and control cells were stained with acridine orange/ethidium bromide and cell morphology was examined under a florescent microscope. Deguelin treatment induced features characteristic of apoptosis, including orange colour, irregular and fragmented nuclei, and irregular cytoplasmic membranes (Fig. 4a and b). Deguelin treatment for 72 h resulted in 85% of the HT-29 cells undergoing apoptosis. Induction of apoptosis was confirmed by detection of digoxigenin-labelled genomic 3′-OH DNA ends (Fig. 4c–f), where a positive effect was noted. After 72 h of treatment with deguelin, approximately 60% of the cells stained positive for apoptosis as determined by this procedure. Internucleosomal degradation of genomic DNA, due to activation of endogenous endonuclease, occurs during apoptosis. Release of oligonucleosome-associated DNA fragments in this process results in a DNA ladder when analysed by agarose gel electrophoresis. Fig. 5 shows that treatment of HT-29 cells with 1 μM deguelin induced DNA laddering after 72 h. Thus, in total these studies demonstrate that deguelin is a potent inducer of apoptosis in these cells.

3.4. Western blot analyses

Since the cell cycle experiments indicated that deguelin exerts its effects by arresting cells in the G1 phase and by inducing apoptosis, we examined changes in the expression of G1 regulatory proteins. Since the progression of the cell cycle is largely controlled by the cyclins, the regulatory units of the cyclin-dependent kinases, we investigated the levels of cyclin E, a cyclin that is highly expressed in the G1 to S phase transition. As shown in Fig. 6, treatment with deguelin (1 μM) for 48 h resulted in reduced levels of cyclin E (34%), with continued downregulation of cyclin E observed over 72 h of exposure with deguelin (20%). Similar downregulation of cyclin E expression was evident for the SW480 and SW620 cell lines (data not shown).

Since deguelin decreased the expression of cyclin E protein in HT-29 cells, it was of interest to determine the protein levels of the cyclin-dependent kinases (CDK) involved in the G1 phase. Cyclin-dependent kinases, including CDK2, CDK4, CDK6, are critical regulators of G1 to S phase transitions. Whereas deguelin had no effect on the protein levels of CDK2. Although no difference is noticed in CDK4 expression

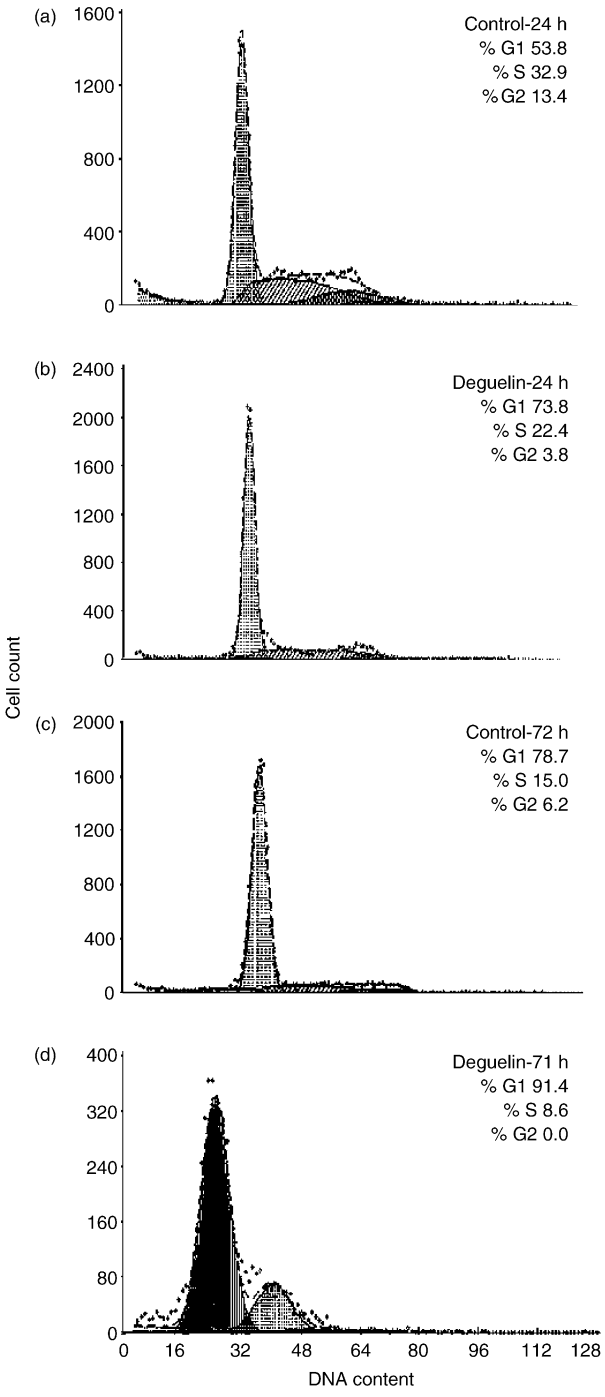

Fig. 3. Effects of deguelin on HT-29 cell cycle distribution. Cell cycle analysis was performed as described in Materials and methods section. Panel a-Control 24 h; Panel b-Deguelin, 24 h. Panel c-Control, 72 h; Panel d-Deguelin 72 h.

at 18 h, values corrected for actin showed a modest 10% reduction in CDK4 expression at this time point. Exposure to deguelin for 24 h resulted in a pronounce decrease of approximately 80% in CDK4 protein levels compared with control cells. Similar downregulation of

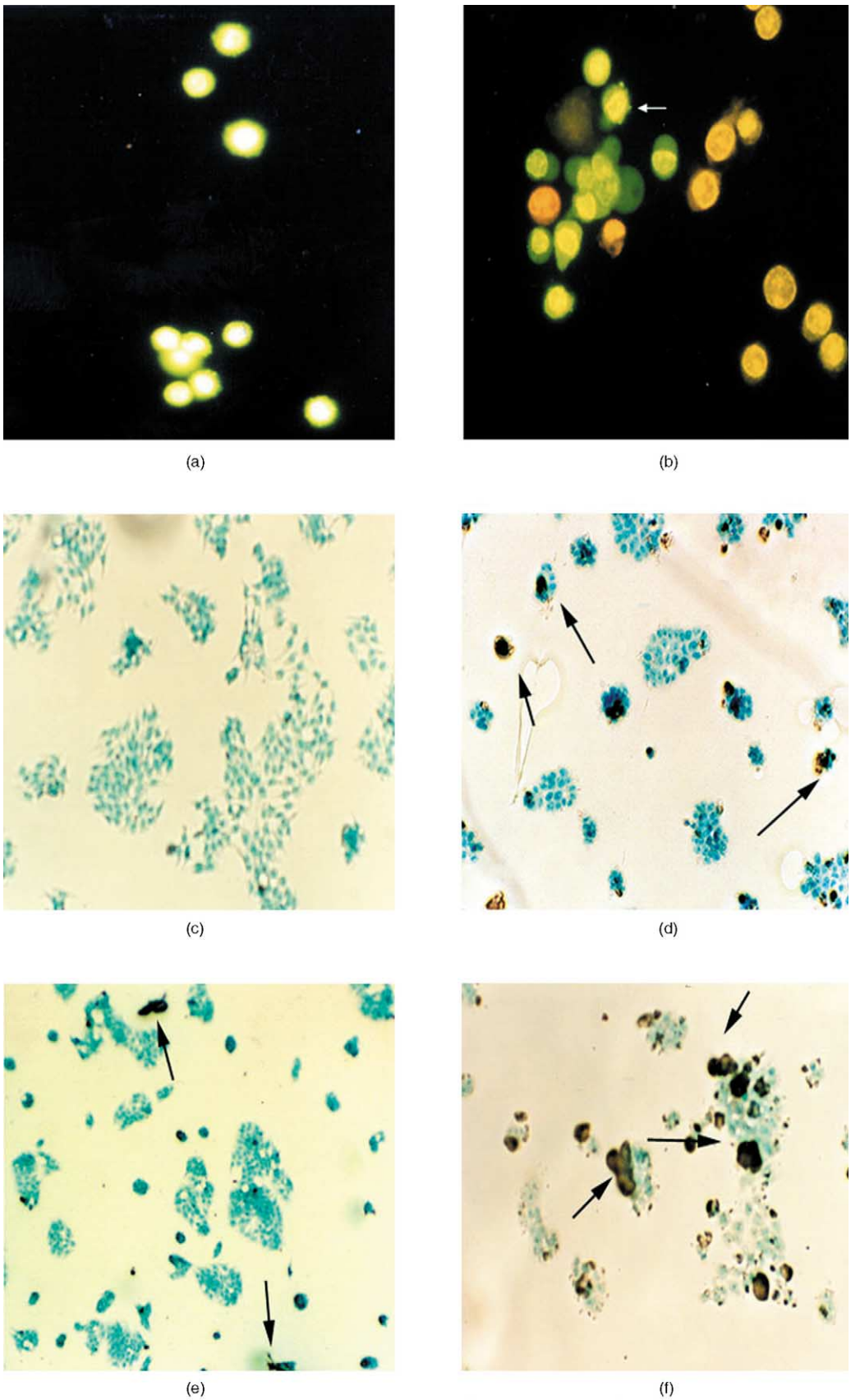

Fig. 4. Apoptosis in HT-29 cells was evaluated by Acridine-orange/ethidium-bromide staining [a] control, [b] deguelin treated for 48 h and by ApopTag [c] control, [d] deguelin treated for 48 h, [e] control, [f] deguelin treated for 72 h. Arrows represent cells exhibiting apoptosis.

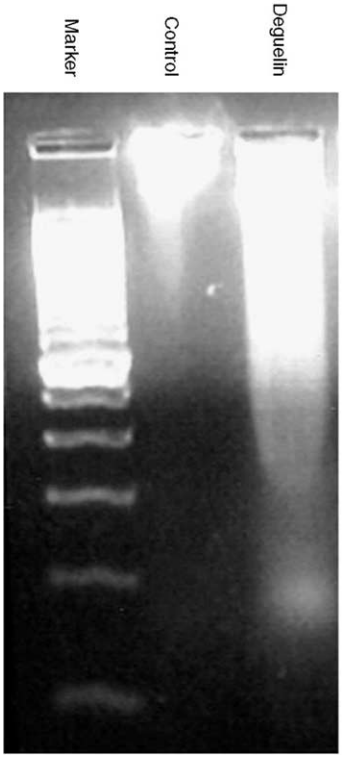

Fig. 5. DNA fragmentation in HT-29 cells treated with deguelin (1 μM) for 72 h. DNA was isolated as described in Materials and methods section.

CDK4 was noted at 48 h (66%) and 72 h of treatment (80%). Expression of PCNA, a protein involved in cell cycle regulation, DNA synthesis and DNA repair, was not affected by deguelin.

We also examined the effects of deguelin on the expression of one of the major cell cycle inhibitor protein, p27, a member of the kinase inhibitor protein family. Results showed that the treatment with deguelin markedly increased the expression of p27 at the 24 h time point (42%). The expression of p27 remained upregulated for 72 h. Similar upregulation was also observed for the SW480 and SW620 colon cancer cells (data not shown). These results suggest that deguelin may be modulating the G1 arrest, at least in part, via an upregulation of p27.

Since the G1 to S phase traverse is generally controlled by the retinoblastoma susceptibility protein (pRb), Western blot analysis for pRb was performed on control and deguelin-treated cells. We observed that the treatment with deguelin resulted in the hypophosphorylation of pRB. These results are consistent with the G1-S-phase block (Fig. 7). Furthermore, Western blot analysis of E2F1, the transcription factor which is responsible for activating genes necessary for S-phase initiation [16], showed a downregulation of protein expression levels by 35% at 24 h, and a decrease of 80% was evident after 48 h of treatment. These results suggest that deguelin may be exerting its effects by regulating the phosphorylation of the Rb, and thus increasing binding and subsequent inactivation of E2F.

4. Discussion

Current evidence suggests that cancer progression may involve the loss of cell cycle checkpoint controls that regulate passage through the cell-cycle. These checkpoints monitor the integrity of the DNA and ensure that genes are expressed in a coordinated manner [17]. By complementing existing cell cycle machinery with extrinsic cell cycle regulators, it may be possible to block the initiation or progression of cancer. Many cancer chemopreventive agents, including naturally occurring and synthetic compounds, are studied for their *in vivo* and *in vitro* anti-tumour efficacy. Previously, we have shown that deguelin effectively inhibits chemically-induced skin and mammary tumours in animal models [11]. In the current report, we have demonstrated a profound apoptotic and anti-proliferative effect of deguelin in HT-29 cells related to growth arrest associated with a G1 phase cell-cycle block. Similar growth arrest in G1 phase was observed for the SW480 and SW620 colon cancer cell lines (data not shown).

Abnormalities of G1 to S phase checkpoint regulators have been recognised as significant factors in the development of human cancers, including colon cancer [18]. Important elements in this regulatory cascade include the cyclin-dependent kinase inhibitor p27, the retinoblastoma protein, and the proliferation promoting transcription factor E2F1. The cell cycle regulator protein p27 has been associated with the prognosis of several cancers, including colon cancer [19], induction of apoptosis in cancer cells [20], and resistance to cancer chemotherapy [21]. Reduced expression of p27 has been observed in several human cancers, such as breast, oesophagus and colon, while p27 knockout mice develop multi-organ hyperplasia and parathyroid tumours [22–24]. Overexpression of p27, on the other hand, has been identified as a crucial element leading to G1 arrest in colon cancer cells treated with agents such as ketoconazole, butyrate and mevastatin [25,26]. These results are consistent with our finding of increased p27 expression after deguelin treatment in HT-29 cells.

Passage through G1 into S phase is regulated in part by the cyclin E/CDK2 complex which serves to hyperphosphorylate the tumour suppressor pRB, leading to liberation of the E2F transcription factor that transactivates genes involved in S-phase progression [16]. Thus, hypophosphorylation of pRb has been associated with inhibition of growth and arrest of cells in the G1 phase [27]. As revealed by Western blot analysis, deguelin treatment resulted in the dephosphorylation of pRb.

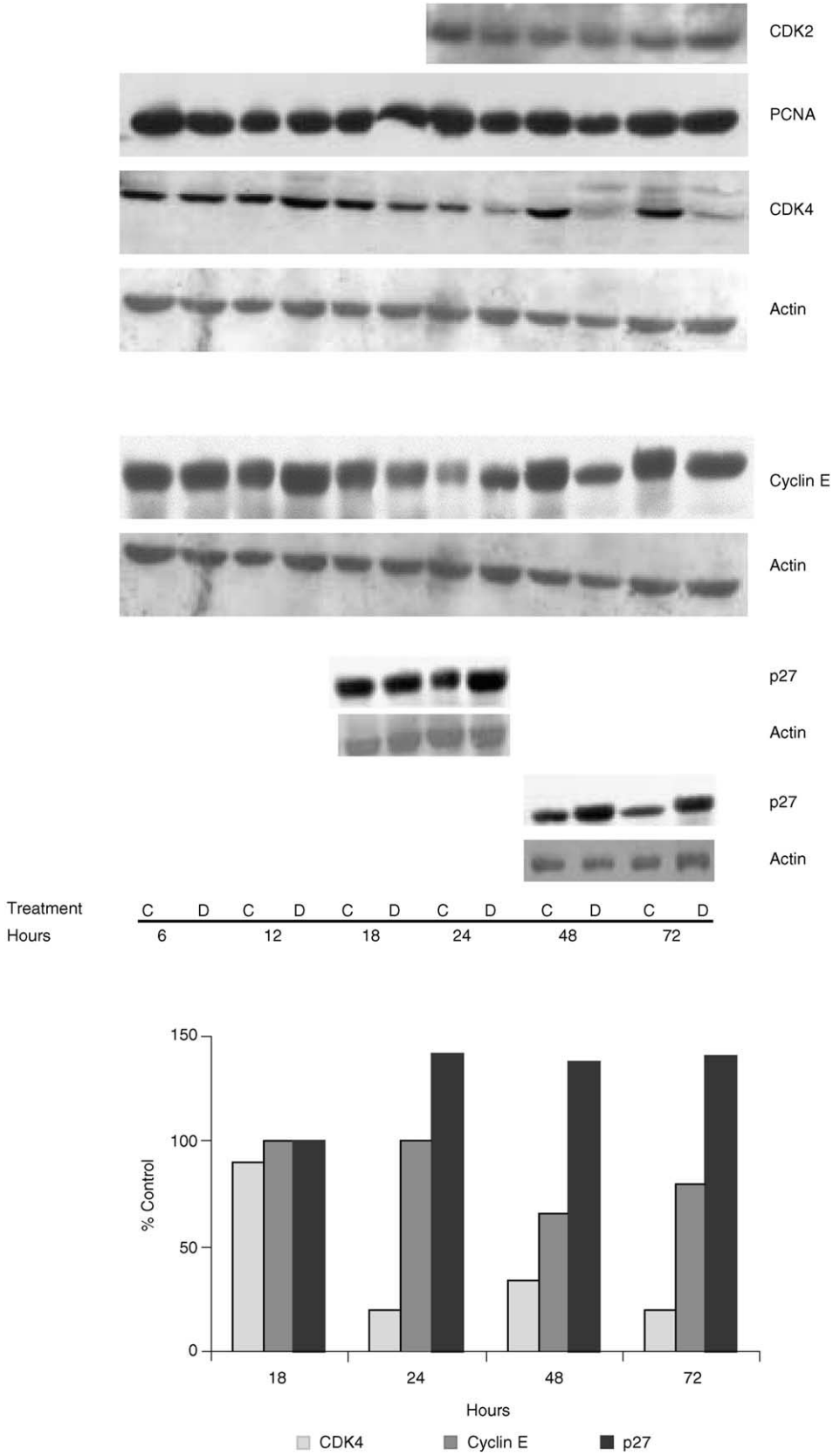

Fig. 6. [a] Western blot analysis in HT-29 cells treated for 6–72 h with [C] vehicle, [D] deguelin (1 μM). Equal loading for each experiment is represented by separate actin bands. [b] Percent change in expression from control cells. The entire experiment was repeated twice with similar results.

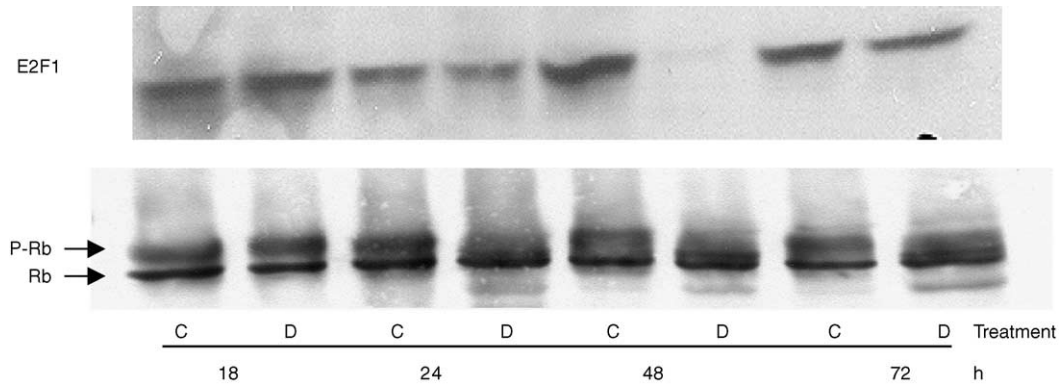

Fig. 7. Western blot analysis of E2F1 and pRb in deguelin-treated cells. C. Control, D. 1 μM deguelin. Exposure to deguelin resulted in a significant increased proportion of the hypophosphorylated form of Rb by 24 h.

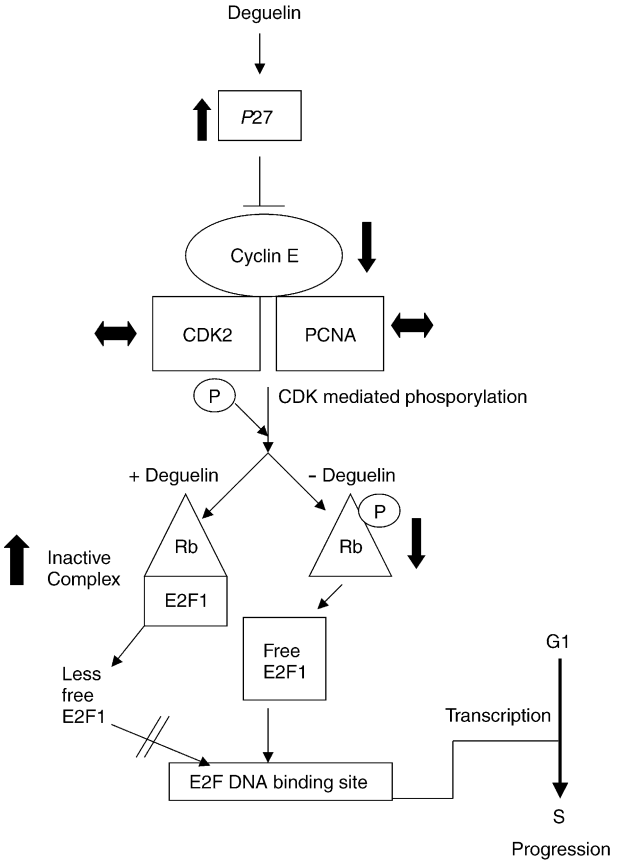

Fig. 8. Schematic representation of the suggested effects of deguelin on the G1 regulatory proteins. Deguelin induces growth inhibition associated with a G1-S-phase block. Thick arrows (↑↓) represent deguelin-mediated effects.

When hypophosphorylated pRb binds to E2F1, it prevents the release and activation of E2F1. A decrease of E2F1 was evident after 48 h of treatment. Therefore, these results suggest that deguelin may cause a G1 arrest by inhibiting the pathways which regulate the phosphorylation of pRB and the subsequent release of E2F1, a crucial step in the G1 to S phase transition. A schematic representation of the suggested effects of deguelin on the G1 regulatory proteins in HT-29 colon cancer cells is shown in Fig. 8. Although this remains speculative at this stage, it suggests a possible mechanism of deguelin action in colon cancer cells. Nonetheless, it suggests a possible mechanism of deguelin action in colon cancer cells.

In the present study, we also demonstrated that deguelin induces apoptosis in colon cancer cells. Apoptosis is a vital element in maintaining tissue homeostasis and is regarded as an important pathway to eliminate unwanted cells [28]. In the colon, elimination of transformed cells via apoptosis has been reported to be a crucial step in the restoration of normal epithelial growth [29]. In recent years, chemotherapeutic agents causing apoptosis have been increasingly appreciated as ideal compounds for the management of cancer [30]. In our studies, we have demonstrated the presence of a sub-G1 peak in flow cytometry, acridine/orange ethidium bromide staining, detection of digoxigenin-labelled genomic 3′-OH DNA ends, and DNA laddering, indicating that deguelin may be potentially mediating its actions by inducing apoptosis in these cells. These data support the potent apoptotic effects of deguelin on colon cancer. Further studies, which examine the mechanism(s) of apoptosis, are warranted.

In conclusion, results described in this report suggest that cell-cycle arrest may, at least in part, underlie the growth inhibitory actions of deguelin observed in HT-29 cells. Therefore, deguelin may serve as a non-toxic adjuvant chemotherapeutic agent in colon cancer.

Acknowledgements

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