Antiproliferative effects of dehydrocostuslactone through cell cycle arrest and apoptosis in human ovarian cancer SK-OV-3 cells

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Abstract. The present study was conducted to investigate the effects of dehydrocostuslactone on the cell cycle distribution and apoptosis of human ovarian cancer SK-OV-3 cells and explored the mechanisms underlying these effects. Dehydrocostuslactone significantly inhibited cell proliferation in a dose-dependent manner and produced significant cell cycle arrest at the G_2/M interface when applied at its IC_{50} (10.7 μM) for this system. Under the same conditions, dehydrocostuslactone caused a slight decrease in the expression of the cell cycle regulatory proteins CDK4 and cyclin E, as well as a small increase in the expression of the cyclin-dependent kinase inhibitor $p21^{Cip1}$. In addition, the dehydrocostuslactone-induced accumulation of cells at the G_2/M phase transition interface resulted in a significant decrease in CDK1 together with cyclin A and cyclin B. This cell cycle arrest induced apoptosis, as confirmed by annexin V and DAPI staining. Following exposure to dehydrocostuslactone, there was a marked increase in the expression of the apoptotic protein Bax and the downstream target p53, a tumor suppressor transcription factor protein, causing the release of cytochrome c. Based on our findings, the mechanism by which dehydrocostuslactone causes cell cycle arrest is via CDK1 down-regulation, and its induction of apoptosis appears to be related to the activation of p53 and the release of cytochrome c.

Introduction

Sesquiterpene lactones occur widely in plants and are regarded as a major class of natural products. Their mechanisms of action are not yet well understood because they are numerous and have a broad spectrum of biological activities (1-4). Recently, some sesquiterpene lactones have been studied for their anticancer activity (5).

Dehydrocostuslactone is one of the main sesquiterpene lactones found in *Saussurea lappa*, a traditional herbal medicine that has been used for cancer treatment in India and Japan (6). *Saussurea lappa* has several biological effects, including anti-inflammatory activities (7-9). It has also been reported that *S. lappa* induces G_2 arrest in human cancer cells, which may be correlated with the induction of apoptosis (10,11).

Until recently, the molecules responsible for the therapeutic activity of *S. lappa* had not been identified. Recent studies have shown that dehydrocostuslactone inhibits the growth of several human cancer cell lines (12-14), but the cellular mechanisms underlying the action of dehydrocostuslactone in the induction of cell cycle arrest and apoptosis remains unknown. Therefore, the aim of the present study was to use the human ovarian cancer SK-OV-3 cells to investigate the cellular mechanisms underlying cell cycle arrest and apoptosis induced by dehydrocostuslactone.

Materials and methods

The cell culture and dehydrocostuslactone treatment. Human ovarian cancer SK-OV-3 cells were purchased from the KCLB (Korean Cell Line Bank, Korea). Cells was routinely maintained in RPMI-1640 [Invitrogen (Molecular Probes), Gibco, Carlsbad, CA, USA], supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 μg/ml streptomycin, Gibco) at 37˚C in a humidified atmosphere containing 5% CO_2. For cell proliferation assay, cells were treated with dehydrocostuslactine ranging from 1 to 100 μM and incubated for 24 and 48 h. To analyze the cell cycle and apoptosis, cells were treated with dehydrocostuslactine at its IC_{50} concentration. Dehydrocostuslactone was purchased from Wako (Wako Pure Chemical Industries, Ltd., Japan) and dissolved in DMSO (final concentration 0.1% in medium).

Cell proliferation assay. Cell proliferation was determined using the MTT assay. At 24 and 48 h point, the cells exposed

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to dehydrocostuslactone were added to methyl thiazolyl tetrazolium (MTT). Four hours later, DMSO was added to each well to dissolve the resulting formazan crystals and then absorbance was recorded at 490 nm in a microplate reader (SpectraMax Plus; Molecular Devices). The value of IC_{50} (i.e., the concentration of the extract required to inhibit cancer cell proliferation by 50% of the control level, each cell type treated with only compound solvent) was estimated from the plot.

Cell cycle analysis. Cells were then harvested, washed with cold PBS, and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at -20˚C for later analysis. The fixed cells were centrifuged at 1,000 rpm and washed with cold PBS at twice. RNase A (20 μg/ml final concentration) and propidium iodide staining solution (50 μg/ml final concentration) was added to the cells and incubated for 30 min at 37˚C in the dark. The cells were analyzed a FACS Calibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software. ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Annexin-V assay. For annexin-based FACS analysis, cells were trypsinized, washed twice in ice-cold PBS, and resuspended in 500 μl binding buffer (Sigma). Annexin V and propidium iodide solution were added to the cell preparations and incubated for 25 min in the dark. Binding buffer (400 μl) was then added to each tube and the samples were analyzed by a FACS Calibur instrument equipped with CellQuest 3.3 software.

DAPI staining assay. Apoptotic morphological changes were determined by DAPI (4',6-diamidino-2-phenyl-indole) staining. After harvesting the cells exposed with apigenin for 72 h, the cells were seeded in poly-l-lysine coated slides and fixed with 4% methanol-free formaldehyde solution for 30 min. Then mounting medium with DAPI (Molecular Probes, Eugene, OR, USA) was dispersed over the entire section of slides. Mounted slides were stored at 4˚C without light. Each slide was observed under Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc., USA).

Immunoblot assay. Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris, pH 7.5) containing protease inhibitor for 1 h at 4˚C. The supernatant was separated by centrifugation, and protein concentration was determined by Bradford protein assay kit II (Bio-Rad Laboratories, CA, USA). Proteins (25 μg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45 μm). The membranes were blocked with a 1% BSA solution for 3 h and washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4˚C. Antibodies against CDK1, CDK2, CDK4, cyclin A, cyclin B, cyclin D, cyclin E, $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, p53, Bax, Bcl-2, cytochrome c, zymogen caspase-3, PARP and ß-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA) and used to probe the separate membranes. The next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-Rad).

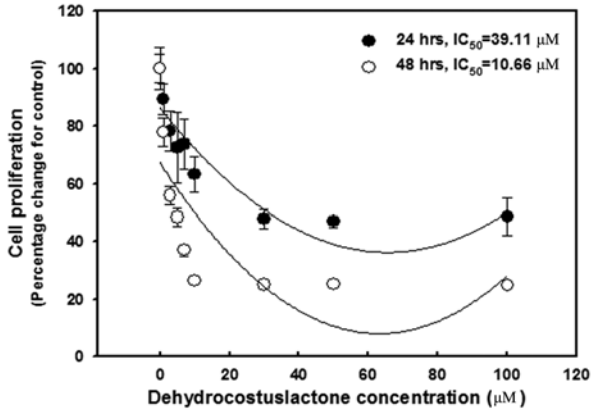

Figure 1. Effect of dehydrocostuslactone on cell proliferation of SK-OV-3 cells. Cells were exposed to either vehicle (0.1% DMSO in medium) or dehydrocostuslactone (1-100 μM) and incubated for 24 and 48 h. All data are reported as the percentage change in comparison with the vehicle-only group, which were arbitrarily assigned 100% viability. *P<0.05, significantly different from the vehicle-only group (dehydrocostuslactone concentration, 0).

Statistical analyses. All data were expressed as percent compared with vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (Sigma Stat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at P<0.05.

Results

Dehydrocostuslactone inhibites cell proliferation. The effects of dehydrocostuslactone on cell proliferation were measured with the MTT assay, using human ovarian cancer SK-OV-3 cells exposed to between 1 and 100 μM dehydrocostuslactone for 24 and 48 h. Dehydrocostuslactone significantly decreased cell proliferation in a time-dependent manner (P<0.05, Fig. 1). Statistical differences in cell proliferation were first exhibited as inhibited cell proliferation at 3 and 1 μM after 24 and 48 h of dehydrocostuslactone treatment, respectively. In addition, dehydrocostuslactone exhibited antiproliferative effects against human ovarian cancer SK-OV-3 cells with the IC_{50} values of 39.11 and 10.66 μM at 24 and 48 h, respectively.

Dehydrocostuslactone arrests the cell cycle at G_2/M phase. To investigate whether dehydrocostuslactone causes cell cycle arrest and apoptosis in SK-OV-3 cells, the cells were exposed to dehydrocostuslactone at its IC_{50} (10.7 μM for 48 h). Compared with vehicle-treated SK-OV-3 cells (control), dehydrocostuslactone-treated cells showed a decrease in the number of cells in G_1 phase (24.2% of the cell population compared with 51.4% for the controls), and this decrease was accompanied by an increase in the number of G_2/M phase

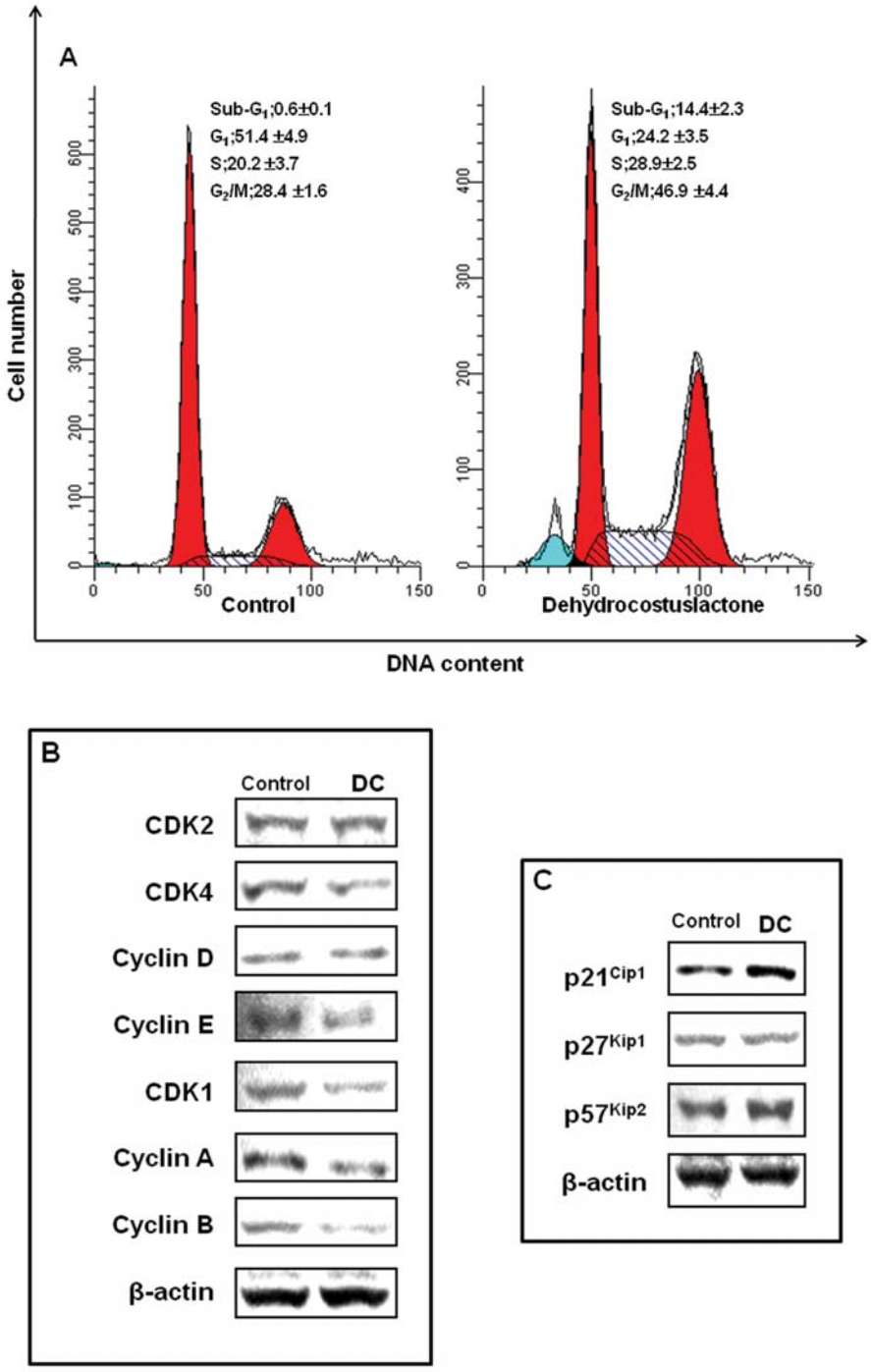

Figure 2. Effect of dehydrocostuslactone on cell cycle distribution and its related regulator protein expression of SK-OV-3 cells. Cells were exposed to either vehicle (0.1% DMSO in medium) or dehydrocostuslactone 100 μM and incubated for 48 h. Values are expressed as percentage of the cell population in the G1, S, and G1/M phase of cell cycle. *P<0.05, significantly different from the vehicle-only group (dehydrocostuslactone concentration = '0').

cells (46.9% of the cell population compared with 28.4% for the controls). This arrest in cell cycle was accompanied by a concomitant decrease in the cell population in G_0/G_1 phase.

Next, we examined the specific regulatory proteins responsible for cell cycle arrest under the same conditions. Compared with control SK-OV-3 cells, the cells treated with dehydrocostuslactone exhibited a slight decrease in the expression of CDK4 and cyclin E and no detectable changes in CDK2 or cyclin D expression (Fig. 3A). However, there was a marked decrease in the expression of CDK1 as well as cyclin A and cyclin B, which combine with CDK1 in controlling the G_2/M transition (Fig. 3A). These results imply that dehydrocostuslactone targets several components of the cell cycle regulatory apparatus. In addition, we examined the expression of inhibitors of cyclin-dependent kinases, including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. Dehydrocostuslactone significantly induced the expression of $p21^{Cip1}$ (Fig. 3B).

Dehydrocostuslactone induces apoptosis. We used annexin-based flow cytometric analysis to detect apoptosis caused by

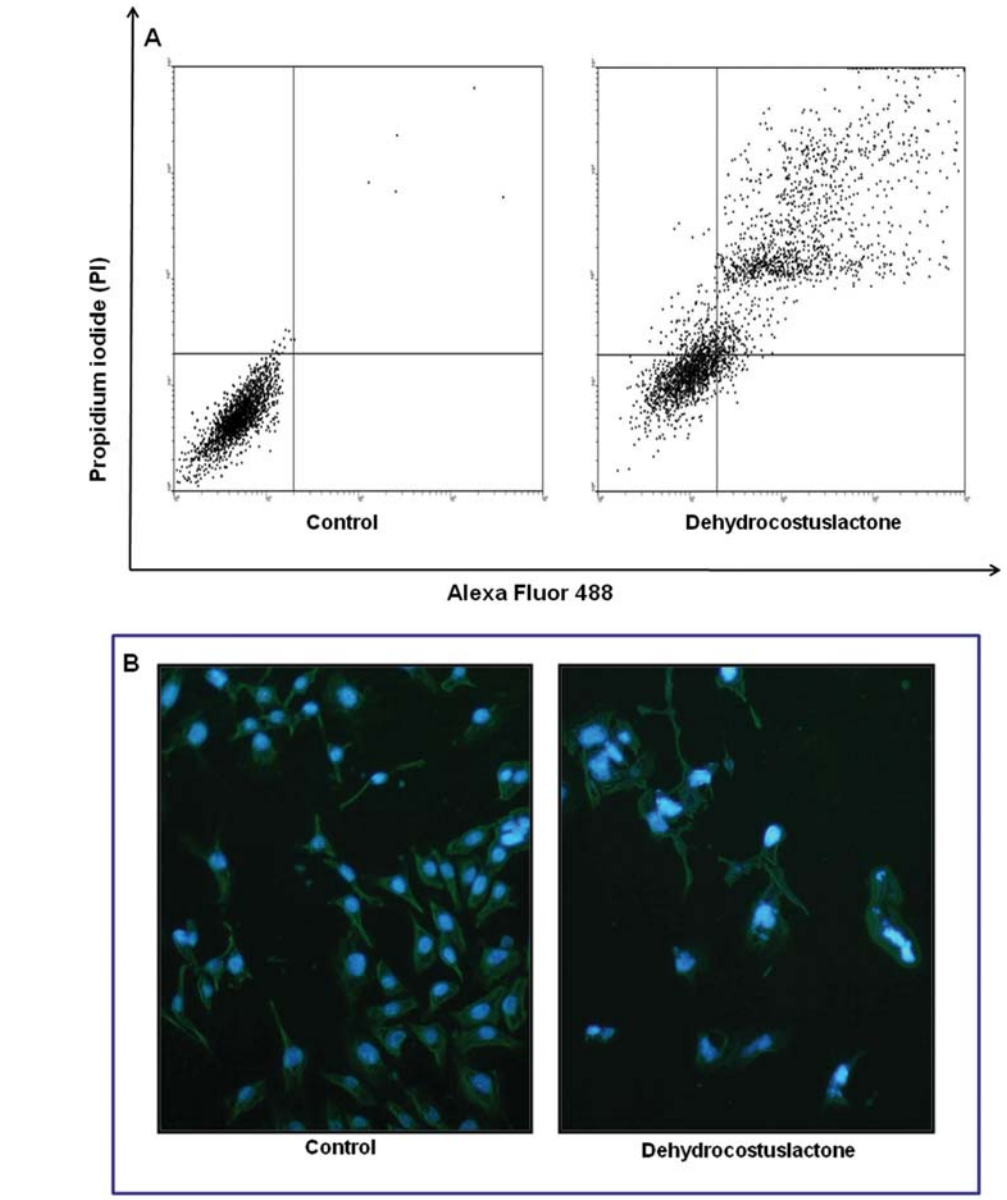

Figure 3. Dehydrocostuslactone induced apoptosis of SK-OV-3 cells. Cells were exposed to either vehicle (0.1% DMSO in medium) or dehydrocostuslactone 100 μM and incubated for 48 h. (A) Apoptotic population was determined by Annexin-V assay. (B) To observe the apoptotic morphological changes, cells exposed to apigenin were fixed and stained by DAPI. (C) Apoptosis related protein expression.

dehydrocostuslactone (Fig. 3A). Under the same conditions discussed above, dehydrocostuslactone treatment resulted in a remarkable shift of cell populations compared with the control group. This is consistent with our findings of an increase in small DNA fragments in sub-G_1 phase (Fig. 2A). In addition, dehydrocostuslactone induced the apoptosis as evidenced by that apoptotic morphological feature such as cell shrinkage and dot-shaped nuclear fragments were observed in exposing cells to dehydrocostuslactone (Fig. 3B).

As presented in Fig. 3C, the activation of p53 was observed in cells treated with dehydrocostuslactone. Coordinated changes in apoptosis-related genes were illustrated by the finding that a decrease in Bcl-2 was correlated with an increase in Bax expression in SK-OV-3 cells exposed to dehydrocostuslactone. Cytochrome c was remarkably released, and the precursor caspase-3 and PARP was also affected by dehydrocostuslactone.

Discussion

Natural phytochemical therapies are increasingly accepted as common form of medicine, and epidemiological and experimental studies have demonstrated that traditional herbs have decreased the incidence of certain forms of cancer (15,16). The actions of herbal remedies may be due to their various biological activities, and it is now becoming widely accepted that such botanical components can make an important contribution to human health.

Even though dehydrocostuslactone is the main sesquiterpene commonly found in *S. lappa*, a well-known traditional herbal medicine used for cancer treatment in Asia, studies have not been conducted on the anticancer activity of dehydrocostuslactone. In the present study, we first examined the antiproliferative effects of various concentrations (1-100 μM) of dehydrocostuslactone on human ovarian cancer SK-OV-3 cells treated for 24 and 48 h. Dehydrocostuslactone significantly decreased the proliferation of SK-OV-3 cells in a dose-dependent manner (10.7 μM for 48 h).

The results of fluorescence-activated cell sorting of SK-OV-3 cells treated with dehydrocostuslactone at its IC_{50} showed that dehydrocostuslactone induced significant cell cycle arrest at the G_2/M interface, suggesting this as a mechanism for the antiproliferative effect of dehydrocostuslactone. These findings are consistent with previous reports that *S. lappa* treatment induced apoptosis and G_2 arrest in a dose- and time-dependent manner via the regulation of cyclins (10).

Cell cycle arrest subsequently induced the down-regulation of cell cycle regulator protein expression in SK-OV-3 cells exposed to dehydrocostuslactone. The cell cycle is tightly mediated through a complex network of positive and negative cell-cycle regulatory molecules such as cyclin-dependent kinases (CDKs), CDK inhibitors (CKIs), and cyclins. G_1-phase progression and G_1/S-phase transition are regulated by CDK2 and CDK4, which assemble with cyclin E and cyclin D. The activated CDK-cyclin complexes are inactivated by binding to CDK inhibitory subunits (CKIs), of which $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ have preferences for CDK2- and CDK4-cyclin complexes (17,18). In the present study, dehydrocostuslactone caused a slight decrease in CDK 4 and cyclin E expression, together with a slight increase in $p21^{Cip1}$. In contrast, the dehydrocostuslactone induced a marked down-regulation of CDK1 (Cdc2) as well as cyclins A and B, which may be the primary cause of G_2/M arrest. CDK1 is a catalytic subunit of the M-phase promoting factor, which is activated at the G_2/M transition and controls the onset of mitosis. Several investigators have shown that CDK1 in combination with cyclin A and cyclin B is critical in the G_2/M phase transition (19,20). The altered pattern of cell cycle-related regulator protein expression induced by dehydrocostuslactone treatment is consistent with arrest at G_2/M because these proteins are not expressed in resting cells. Thus, G_2/M arrest via CDK1 down-regulation may be an important molecular mechanism by which dehydrocostuslactone inhibits cancer cell growth.

The inhibition of cell growth and the cell cycle arrest induced by dehydrocostuslactone led in turn to apoptosis, as confirmed by annexin/propidium iodide staining and the sub-G_1 peak assessed by flow cytometry. Under the same conditions, dehydrocostuslactone also induced well-known apoptotic morphological features such as membrane blebbing.

The tumor suppressor gene p53 is regarded as a key factor in the balance between cell survival and cell death via the regulation of both the G_1 and G_2/M phases of the cell cycle (21). The activation of p53 in response to DNA damage led to cell cycle arrest and the inhibition of cell proliferation (22-24).

Dehydrocostuslactone significantly activated the expression of p53. The release of cytochrome c from mitochondria to the cytoplasm is a key step in the initiation of apoptosis. Dehydrocostuslactone-induced cytochrome c release was activated by caspase-3 and poly(ADP_ribose) polymerase (PARP), as evidenced by a decrease in zymogen caspase-3 and PARP expression. As the release of cytochrome c from mitochondria to the cytoplasm is a key step in the initiation of apoptosis, cytochrome c release by dehydrocostuslactone was activated the caspase-3 and PARP evidenced by a decrease of zymogen caspase-3 and PARP expression.

In conclusion, we suggest the existence of multiple pathways by which dehydrocostuslactone treatment results in G_2/M cell cycle arrest, via the down-regulation of CDK1 together with cyclins A and B, and apoptotic cell death, via modulation of the p53 pathway and up-regulation of cytochrome *c* expression. Although the mechanism of its anticancer activity was investigated only in an *in vitro* cell system, dehydrocostuslactone has shown potent anticancer activity *in vivo* and is an excellent candidate for further study *in vivo*.

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