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Deoxyelephantopin inhibits cancer cell proliferation and functions as a selective partial agonist against PPARγ

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ABSTRACT

Deoxyelephantopin (ESD) was reported to potentiate apoptosis, inhibit invasion and abolish osteoclastogenesis but no target protein was disclosed. Here, we discovered that ESD could significantly inhibit the proliferation of different cancer cells and induce apoptosis and cell cycle arrest at G_2/M phase in HeLa cell. Moreover, biochemical and biophysical assays revealed that ESD acted as a specific partial agonist against PPARγ. Molecular docking with site-directed mutagenesis analyses indicated that ESD functioned as a partial agonist of PPARγ by adopting a distinct binding mode to PPARγ compared with rosiglitazone. The PPARγ knockdown results indicated that the inhibition of ESD against the cancer cell proliferation is more possibly through PPARγ-independent pathway and our findings might supply potent binding features for ESD/PPARγ interaction at atomic level, and shed light on the potential acting target information for this natural compound.

1. Introduction

It has been known that the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPARγ) is related not only to metabolic disorders but also to inflammation, atherosclerosis, immunomodulation and cancer [1]. The four somatic PPARγ mutations found in sporadic colon cancers have suggested that PPARγ might act as a tumor suppressor [2] as further confirmed by the fact that synthetic PPARγ full agonists, thiazolidinediones (TZDs) (e.g. troglitazone, Fig. 1) were able to induce cell differentiation and apoptosis or inhibit cell proliferation both *in vitro* (e.g. human liposarcoma [3], colon cancer [4] and malignant breast epithelial cells [5,6]) and *in vivo* (e.g. female triple-immunodeficient BNX nude mice (Harlan–Sprague–Dawley) [5,6]).

To date, a number of reports have indicated that the therapeutic benefits of PPARγ agonists might go far beyond their uses in diabetes, and evidence has emerged of their uses

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Abbreviations: ESD, deoxyelephantopin; PPARγ, peroxisome proliferator-activated receptor-γ; TZDs, thiazolidinediones; NF-κB, nuclear factor-κB; LBD, ligand-binding domain; AF-2, ligand-dependent activation domain; SRC-1, steroid receptor coactivator-1; CBP, cAMP-response element-binding protein (CREB)-binding protein; IPTG, isopropyl-β-D-thiogalactoside; IKK, IκBα kinase; AP-1, activator protein-1; STAT, signal transduction and activator of transcription; HDAC3, (NCoR)-histone deacetylase-3.

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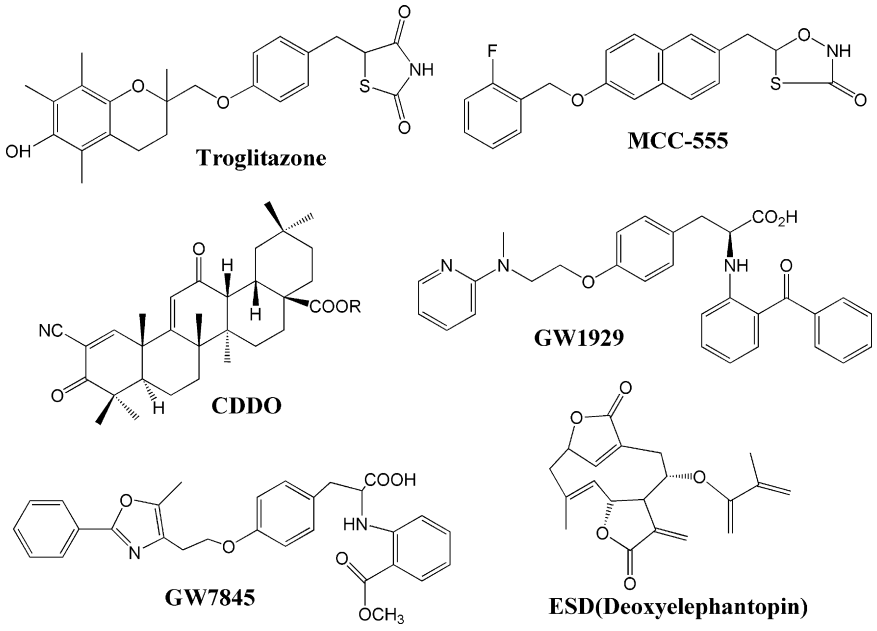

Fig. 1 – Structures of deoxyelephantopin (ESD) and other known PPARγ agonists with anti-tumor activities.

in inflammatory and cancer diseases. Apart from the above-mentioned TZDs, some other types of PPARγ ligands with anti-tumor activities were also discovered (Fig. 1). For example, CDDO induced growth arrest and apoptosis of breast cancer cell by acting as a partial agonist against PPARγ [7]; GW7845, a potent PPARγ agonist, significantly reduced the incidence, number, and weight of tumors when fed to rats after carcinogen administration [8]; MCC-555, a unique partial agonist of PPARγ as an antidiabetic drug, inhibited the growth of prostate cancer cells both *in vitro* and *in vivo* [9,10]; GW1929, one of the PPARγ agonists, could stimulate the differentiation of human neuroblastoma cells [11]. Additionally, several PPARγ antagonists, e.g. GW9662 and T0070907, were also reported to strongly inhibit the growth of squamous cell carcinomas presumably by blocking the PPARγ pathway [12,13]. All these results have thereby offered an exciting prospect of using PPARγ ligands for tumor therapy.

The natural product deoxyelephantopin (ESD, Fig. 1) was recently reported to repress nuclear factor-κB (NF-κB) activation, but its direct target was not disclosed [14]. Here, we discovered that ESD functioned as a partial agonist of PPARγ and could significantly inhibit the proliferation of different cancer cells and caused cell cycle arrest at G2/M phase.

2. Materials and methods

2.1. Reagents

All solvents and reagents were purchased commercially and were used without further purifications. The affinity columns and lower molecular weight (LMW) marker were purchased from Amersham Pharmacia Biotech, isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Promega. SRB and PI were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM), RPMI1640 and MEM medium were from GibcoBRL, and fetal bovine serum (FBS) from HyClone. The anti-PARP antibody was purchased from Beyotime Co., anti-PPARγ antibody was from calbiochem Co., and caspase 3 and caspase 9 antibody were from Santa Cruz Biotechnology, Inc. Lipofectamine 2000 was purchased from Invitrogen. The QuikChange II site-directed mutagenesis kit was from Stratagene.

2.2. Plasmids

The PPARα-LBD and PPARδ-LBD were amplified by PCR from pSG-hPPARα (provided by Dr. X. Lu, Shenzhen Chipscreen Biosciences Ltd.) and pAdTrack-PPARδ (provided by Dr. B. Vogelstein, Howard Hughes Medical Institute, US) respectively, and then subcloned into vector pET15b to express the His-tagged fusion proteins. The pSuperbasic-siPPARγ was constructed by inserting the coding sequence of siRNA into the pSuperbasic with BglII–HindIII sites. The sequences of the small interfering RNA (siRNA) against the PPARγ message was 5′-GCCCTTCACTACTGTTGAC-3′ [15]. The plasmid pET15b-hPPARγ-LBD was kindly donated by Dr. J. Uppenberg, Department of Structural Chemistry, Pharmacia and Upjohn, Sweden, pcDNA3.1-hPPARγ was a gift from Dr. X. Gao (Chengdu Institute of Biology, CAS, China), and the reporter gene pSV-PPRE-Luc was kindly provided by Dr. Ronald M. Evans (The Salk Institute for Biological Studies, La Jolla, CA, USA). The Gal4-PPARγ-LBD expression plasmid and the UAS-E1b-TATA-Luc reporter were generously donated from Prof. J. L. Jameson, Department of Medicine, Northwestern Memorial Hospital.

2.3. Protein expression

Expression and purification of the PPARγ-LBD, PPARα-LBD and PPARδ-LBD proteins were performed according to the published methods [16]. The mutant proteins PPARγ-LBD (S342G)

and PPARγ-LBD (G284A) were purified by renaturing inclusion bodies based on the published approach [17]. The correct refolding of the mutant proteins was confirmed by circular dichroisic spectra (Supplementary Fig. S1).

The proteins were concentrated with a 10-kDa cut-off membrane (Amicon) at 4 °C, and protein concentration was measured by the standard Bradford method.

2.4. Site-directed mutagenesis

Point mutants were introduced into pET15b-hPPARγ-LBD by PCR-based mutagenesis strategy according to the instruction manual (www.Stratagene.com) with the following mutagenic forward primers (residues underlined represent the mutated codon): S342G, 5′-GATGGGGTTCTCATAGGCGAGGGCCAAGGCTTC-3′; G284A, 5′-CCGCATCTTTCAGGCCTGCCAGTTTCGCTC-3′. All the mutations were verified by sequencing.

2.5. Ligand-binding assay

The binding of the related compounds towards PPARγ-LBD, PPARα-LBD, PPARδ-LBD, PPARγ-LBD (S342G) and PPARγ-LBD (G284A) proteins was investigated by use of SPR technology-based Biacore 3000 instrument. During the experiment, the relevant proteins were dissolved in coupling buffer (15 μg ml^{-1}, in 10 mM sodium acetate, pH 5.0) and immobilized on a CM5 sensor chip with *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) according to the standard amine-coupling procedure. The baseline was equilibrated with a continuous flow of running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20) through the chip for 1–2 h. Different concentrations of related compounds were injected into the channels at a flow rate of 30 μl min^{-1} for 60 s, followed by disassociation for 120 s. BIAevaluation software version 3.1 (Biacore) and the 1:1 Langmuir binding fitting model were used to determine the equilibrium dissociation constant (K_D) of ESD (or rosiglitazone) binding to the proteins.

2.6. Molecular modeling

The 3D structure of ESD was constructed by the online demonstration of Corina (http://www.mol-net.com/online demos/corina demo.html). The 3D model of PPARγ-LBD was retrieved from the Brookhaven Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) (PDB ID 2F4B) [18]. AutoDock Tools (http://autodock.scripps.edu/resources/adt) was used to add hydrogen and assign partial charges to both protein and ligand. AutoDock 3.0.5 [19] was employed for the docking of ESD to PPARγ-LBD. All the molecular modeling and docking simulations were performed on a Silicon Graphics Origin3800 (with 128 CPUs).

2.7. Transient transfection assay

Transactivation assay for the full-length PPARγ was performed as previously described [20]. Cells were transiently transfected with expression vectors pSV-PPRE-Luc (0.3 μg), pCDNA3.1-PPARγ (0.2 μg), pCDNA3.1-RXRα (0.2 μg) and pRL-SV40 as internal control indicated by Dual-Luciferase Reporter Assay System (Promega). After transfection for 24 h, cells were placed in phenol red-free Dulbecco's modified Eagle's medium with increasing concentrations of ESD or certain concentration of rosiglitazone containing different concentrations of ESD. After incubation with the above compound for another 24 h, cells were lysed and luciferase activity was determined as the -fold activation relative to the untreated cells after normalization with Renilla luciferase values.

A fusion protein containing the yeast Gal4 DNA-binding domain linked to the ligand-binding domain of PPARγ was also used in transfection experiments with reporter construct UAS-E1b-TATA-Luc as described [21]. All transfections were performed in triplicate and results were expressed as mean ± S.D. Statistical analysis was performed using Student's *t*-test, with $p < 0.05$ considered statistically significant.

2.8. Sulphorhodamine B assay for cell proliferation

The quantitative sulphorhodamine B (SRB) colorimetric assay [22] was used to determine the anti-proliferation activity of ESD against HGC, A549, and HeLa cells. Cells were seeded into a 96-well plate with 5000 cells per well and incubated at 37 °C for 24 h. The cells were treated with increasing concentrations of ESD for another 72 h and then fixed with 10% trichloracetic acid for 1 h at 4 °C, followed by air-drying and staining for 20 min at room temperature with 4 mg/ml SRB solution. After that, cells were washed with 1% acetic acid for 5 times and dissolved with 150 μl of 10 mM unbuffered Tris base. Cell viability was measured by the optical density at 515 nm (Benchmark PlusTM microplate spectrophotometer) and calculated from the data of three wells, the values were obtained from three independent experiments. The wells with or without drugs were used as positive or negative controls.

2.9. RNA interference assay

HGC cells were seeded in 24-well plates and incubated overnight to allow cells to attach to the plate. Transfection was performed according to the manufacturer (www.invitrogen.com). 0.8 μg PPARγ siRNA or the control vector pSuper-basic was transiently transfected into the cells with Lipofectamine 2000 (1 μl per well). Six hours after transfection, the medium was replaced with growth medium containing with serum and the cells were cultured at 37 °C in a CO_2 incubator for another 18 h. ESD (10 μM) was then added and incubated for 24 h. The cell numbers were measured by SRB assay as indicated in the above method.

2.10. Cell cycle and apoptosis analysis

HeLa cells (6×10^4 cells/well) were seeded in six-well plates and incubated overnight to allow cells to attach to the plate. After cells incubated with increasing concentrations of ESD for 24 h, the adherent cells were detached with trypsin and the floating cells were then collected by centrifugation at 600 g. The cells were further washed twice with PBS and fixed in 75% ethanol at 4 °C, and collected by centrifugation. Cells were resuspended with 500 μl PBS containing 100 mg/ml RNase A and incubated for 30 min at 37 °C, followed by filtration and staining with 0.05 mg/ml propidium iodide (PI) for 1 h. The

suspensions were then analyzed by Becton Dichinson FACScan BD Biosciences, San Jose, CA. The percentage of cells in the G_0/G_1, S and G_2/M phases of cell cycle was determined by their DNA contents and presented as fold of control. Apoptosis was measured by PARP cleavage assay and caspase activation based investigation, which was blotted against ESD-treated cells with anti-PARP, anti-caspase 3 and anti-caspase 9 antibodies based on Western blot analysis.

2.11. *Statistical analysis*

The data were presented as mean ± S.D. for at least three separate determinations for each group. Differences between groups were examined for statistical significance using Student's *t*-test. $p < 0.05$ was used to indicate a statistically significant difference.

3. Results

3.1. *ESD is a selective PPARγ ligand as evaluated by SPR assay*

Since PPARγ ligands, e.g. PPARγ agonists, partial agonists and antagonists, were confirmed to bind to the ligand-binding domain of PPARγ *in vitro*, it is essential to investigate the binding of ESD against PPARγ, which is the prerequisite for determining the agonistic or antagonistic feature of ESD towards PPARγ. Here, we used SPR technology based Biacore 3000 biosensor to quantitatively investigate the ESD binding to PPARγ. The equilibrium dissociation constant (K_D) for evaluation of PPARγ binding to ESD or rosiglitazone (as a positive control) was obtained by fitting the sensorgram with a 1:1 (Langmuir) binding fit model. As shown in Fig. 2B, ESD showed high binding affinity against PPARγ-LBD (K_D = 3.4 μM), similar to that for rosiglitazone (K_D = 2.2 μM, Fig. 2A). Moreover, to further inspect the potential binding specificity of ESD against PPARγ, we also examined its binding to PPARα-LBD and PPARδ-LBD. By using GW14643 (PPARα agonist) and benzafibrate (PPARδ agonist) as positive controls (Supplementary Fig. S2A and B), the SPR results clearly indicated that ESD exhibited no binding affinity against PPARα-LBD and PPARδ-LBD (Fig. 2C and D), suggesting that ESD might be a selective ligand of PPARγ.

3.2. *ESD increases PPARγ transactivation*

In the determination of the ESD activation against PPARγ in cultured cells, transactivation assay was performed. In COS-7 cells, ESD could activate the transcription activity of PPARγ in a dose-dependent manner and the maximal efficacy was achieved by 20 μM ESD, as shown in Fig. 3A. Western blot

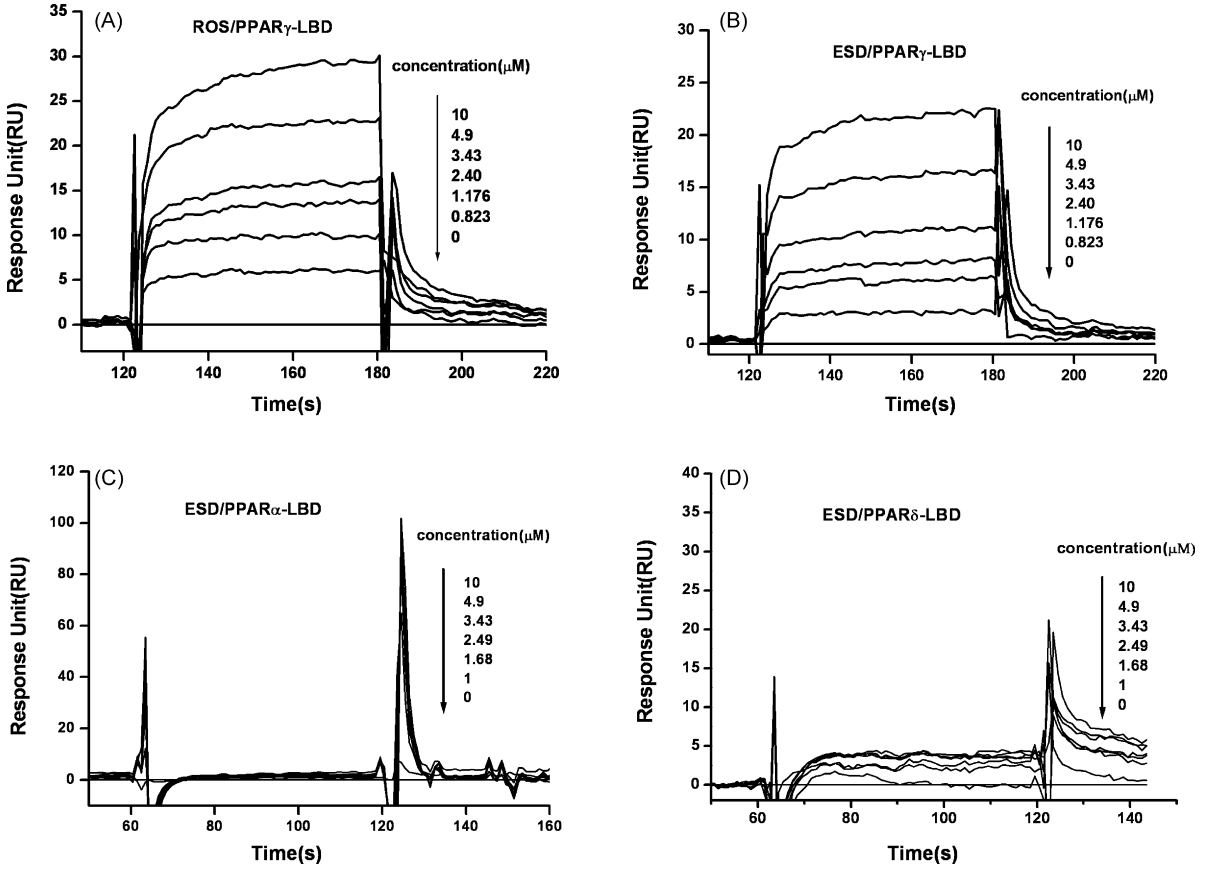

Fig. 2 – Deoxyelephantopin (ESD) specifically binds to PPARγ as investigated by SPR technology based Biacore 3000 instrument. The sensorgrams were obtained from injection of series of concentration of rosiglitazone over the immobilized PPARγ-LBD (A); or ESD over the immobilized PPARγ-LBD (B); PPARα-LBD (C); PPARδ-LBD (D); PPARγ-LBD (S342G) (G) and PPARγ-LBD (G284A) (H), and rosiglitazone over the immobilized PPARγ-LBD (S342G) (E) and PPARγ-LBD (G284A) (F).

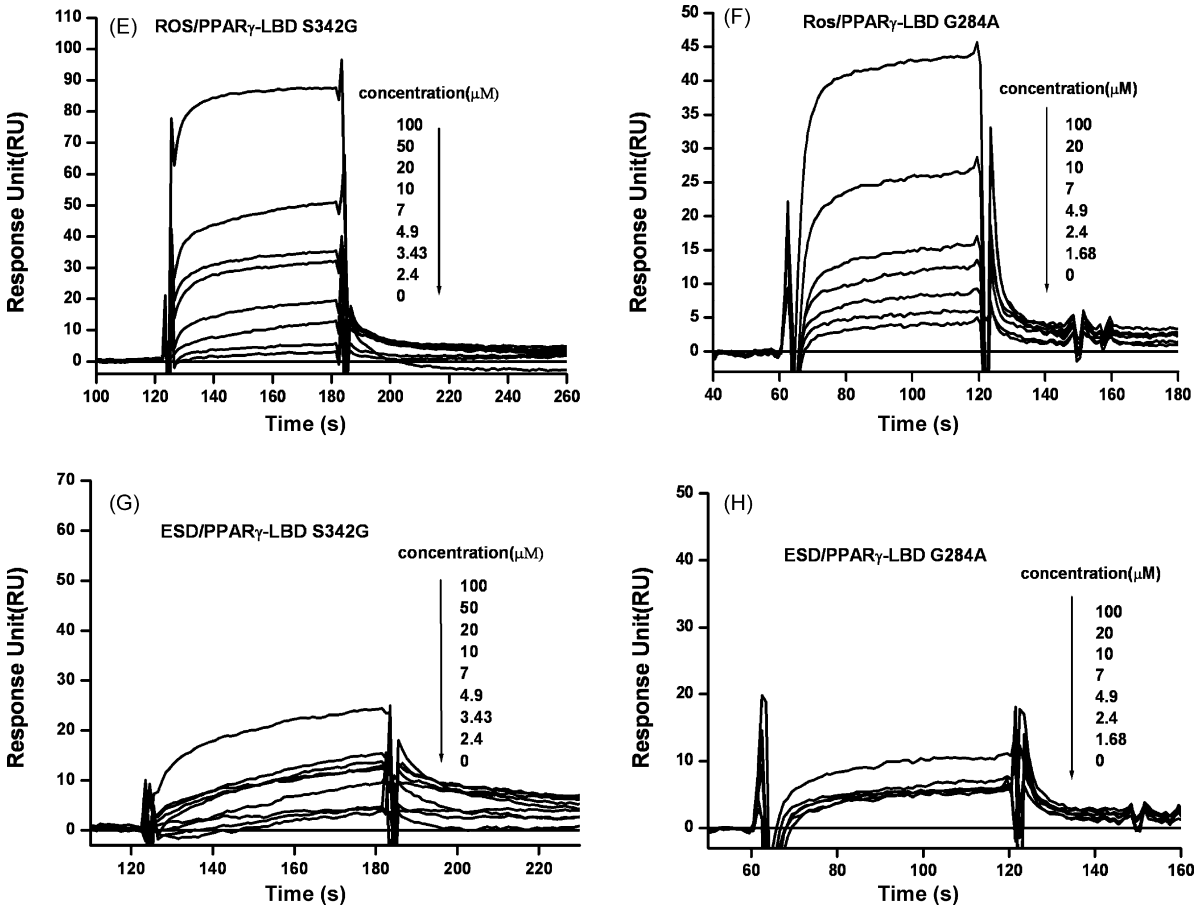

Fig. 2. (Continued).

analysis showed that PPARγ is highly expressed in HGC, HeLa and A549 cells (Supplementary Fig. S3), and ESD could also increase the PPARγ transcription activity in these three cancer cells (Fig. 3D). Moreover, a significant enhancement of PPARγ transcription activity was observed when increasing concentrations of ESD were added to a non-saturating concentration of rosiglitazone (Fig. 3B and D).

To further determine whether ESD activated PPARγ via its ligand-binding domain, we fused PPARγ-LBD with Gal4DNA-binding domain and tested the effect of ESD on this chimeric protein. As indicated in Fig. 3C, ESD could transactivate Gal4-PPARγ-LBD with a comparable efficacy as it activates full-length PPARγ, and also significantly enhance the rosiglitazone's agonistic effect on PPARγ-LBD. These results thus implied that ESD exhibited less potent transactivation activity than rosiglitazone.

3.3. *Molecular modeling of PPARγ-LBD/ESD binding*

Since we have found that ESD could increase PPARγ transactivation by functioning as a selective PPARγ ligand, to further investigate PPARγ-LBD/ESD interaction at atomic level, molecular docking analysis was employed. During the assay, ESD was docked into the ligand-binding site of PPARγ-LBD with the program AutoDock 3.0.5 [19]. The top pose, ranked by the "estimated free energy of binding", was chosen as the predicted binding mode of ESD. As shown in Fig. 4A, ESD bound in the hydrophobic part of ligand-binding pocket of PPARγ-LBD. Twenty-one hydrophobic interaction atom pairs between the compound and the protein formed. Six atom pairs were contributed by the hydrophobic interaction between Gly284 and the large ring (C1, C2, C3, C4, C8, C10, C11, C12, C14 and C15) of ESD (Fig. 4C), which might anchor the compound in the pocket. The lack of side chain of Gly284 seemed to be very important for ESD binding (Fig. 4B), since the side chain would exert steric repulsive effect on this large ring of the compound. As indicated in Fig. 4C, ESD formed only one hydrogen bond with the side-chain oxygen (OG) of Ser342. Additional docking studies of the mutated PPARγ-LBD (G284A and S342G) showed that the docked ESD could no longer be in the ligand-binding pocket of PPARγ-LBD (data not shown). These results thereby indicated that residues Gly284 and Ser342 are essential for ESD binding to PPARγ-LBD.

By structural alignment using Swiss-PdbViewer [23] of the three human PPAR isoforms, it is noteworthy to see that residues [Gly284, Ser342] in PPARγ are substituted by [Cys275, Ala333] in PPARα and [Arg284, Ala342] in PPARδ. Obviously, the side chains of Cys275 in PPARα and Arg284 in PPARδ may exert steric repulsive effects on the large ring of ESD, while Ala333 in PPARα and Ala342 in PPARδ lack hydrogen-bond donor in their side chains. Therefore, such residue substitutions among

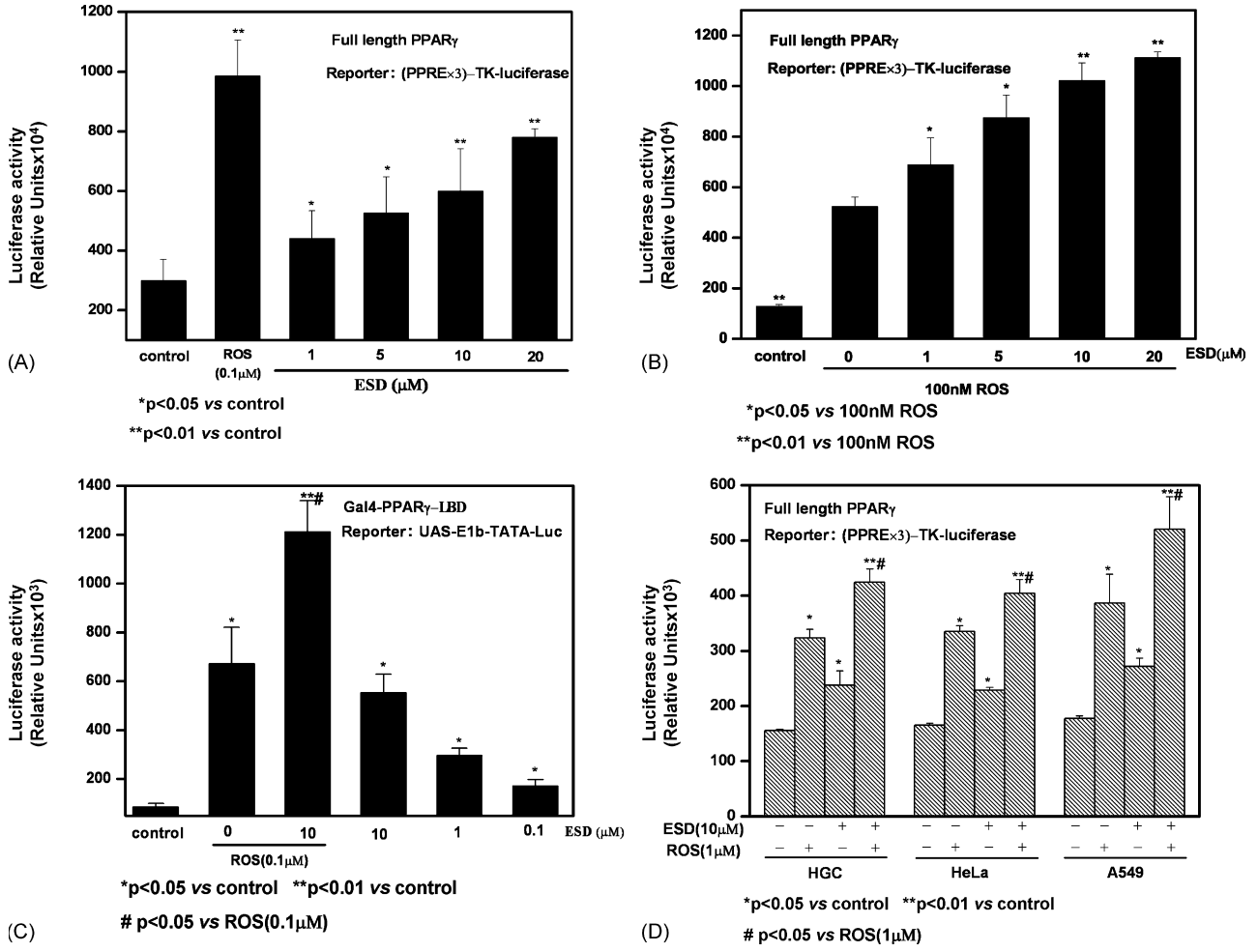

Fig. 3 – Effects of ESD on PPARγ transcription activity. Cos-7 cell was transiently co-transfected with hPPARγ/RXRα/pSV-PPRE-Luc and incubated with different concentrations of ESD (A), or stimulated with a fixed amount of rosiglitazone (10^{-7} M) together with increasing concentrations of ESD (B), and activation of ESD on PPARγ-Gal4/UAS-E1b-TATA-Luc (C). HGC, A549 and HeLa cells were transiently co-transfected with hPPARγ/RXRα/pSV-PPRE-Luc and incubated alone with 10 μM ESD or together with 1 μM rosiglitazone (D). Each point was performed in triplicate, and the figure is a representative of four independent experiments. $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. roglitazone (ROS).

human PPAR subtypes have provided a good explanation for the binding selectivity of ESD against PPARγ.

In addition, the importance of residues Gly284 and Ser342 in PPARγ/ESD binding was further supported by SPR assay against the mutated PPARγ-LBD proteins (G284A and S342G). As shown in Fig. 2G and H, these two mutations abolished the binding of ESD to PPARγ, while rosiglitazone retained highly binding affinity to the mutants (Fig. 2E and F). These results thus confirmed the above-mentioned ESD/PPARγ-LBD interaction model, in which ESD adopted a distinct binding mode compared with rosiglitazone [24].

3.4. *ESD inhibits proliferation of different cancer cells through PPARγ-independent pathway*

Based on the recent reports that PPARγ has been developed as an essential target for exploring potential anti-cancer agents and PPARγ agonists exhibited anti-tumor activities both *in vitro* and *in vivo* [1,6], SRB based assay was thus applied to test the inhibition of ESD against the proliferation of HGC, A549 and HeLa cancer cells in our work. As indicated in Fig. 5A–C, ESD exhibited high inhibition activities against the proliferation of A549, HeLa and HGC cells with IC_{50} values of 4.2, 5.4 and 4.7 μM, respectively. Altogether, the results demonstrated the anti-tumor function of ESD in several different cancer cell lines.

To investigate whether the anti-proliferation effect of ESD on cancer cells could be mediated by PPARγ activation, HGC, A549 and HeLa cells were pretreated with or without the PPARγ specific antagonist GW9662 (20 μM) for 45 min before the addition of ESD (10 μM) for additional 24 h. Cell numbers were determined by SRB assay. As shown in Fig. 5D, ESD significantly decreased cell numbers and GW9662 (20 μM) itself had no detectable effect on cancer cell. The GW9662-pretreatment cancer cells failed to show the abrogation of the inhibitory effect of ESD on cell growth. Moreover, to further study the relationship between the anti-proliferation effect of ESD and PPARγ activation, siRNA technology based assay was

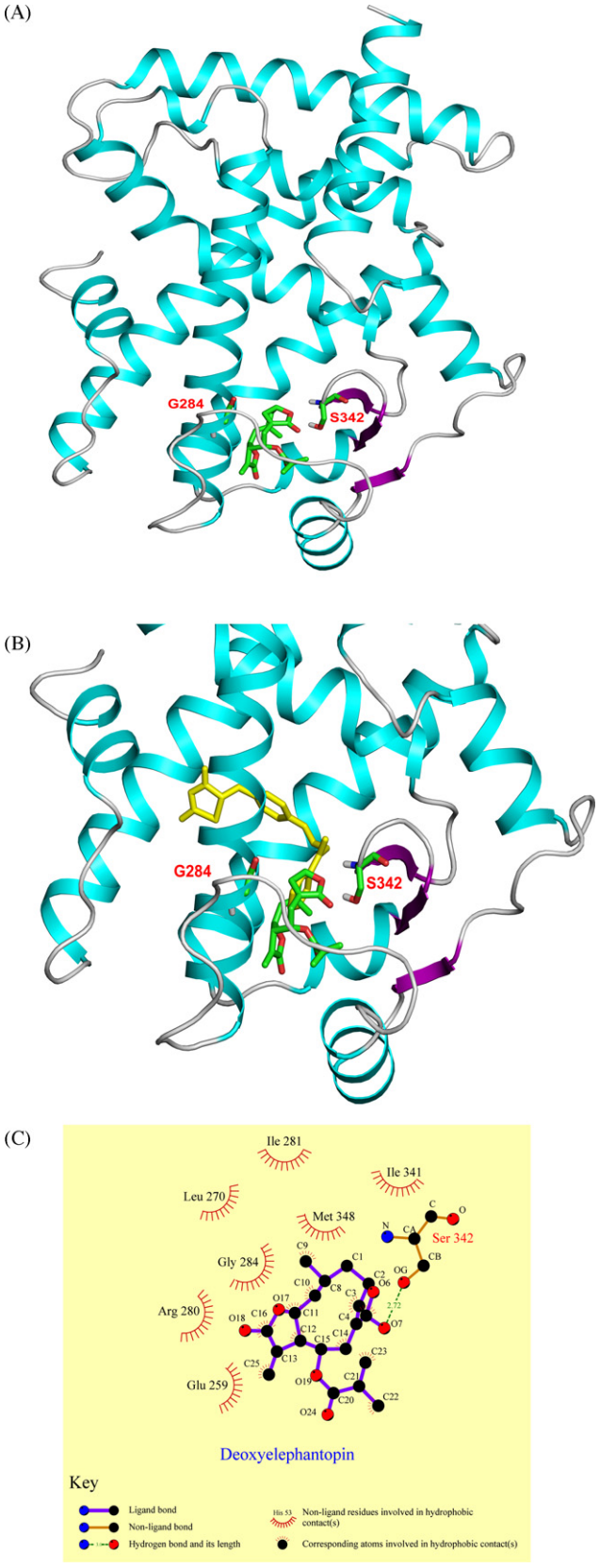

Fig. 4 – Modeled interaction of ESD with PPARγ-LBD. Protein backbone of PPARγ-LBD is shown as a ribbon model. Selected protein residues (Gly284 and Ser342) and ligands are shown as stick models. (A) Model structure of ESD was docked into a crystallographic structure of the PPARγ-LBD (Protein Data Bank entry 2F4B); (B) close-up of the PPARγ ligand binding pocket with ESD, and rosiglitazone (yellow, from PDB entry 2PRG, overlayed by superposition of protein backbone); (C) amino acid residues involved in the ESD binding, H-bond represented as dashed line, and spiked residue hydrophobic contacts with the ligand. (A) and (B) were generated with PyMOL and (C) was generated with LIGPLOT.

also carried out, in which HGC cell was transfected with pSuperbasic (as a control group) and PPARγ siRNA plasmid that caused a decrease in PPARγ protein level (Fig. 5E, inset). As indicated in Fig. 5E, in the PPARγ knockdown HGC cell, the inhibition ability of ESD against the cell growth could not be repressed as compared with the control group (transfection with pSuperbasic). All these results thus implied that ESD could inhibit the proliferation of cancer cells possibly through a PPARγ-independent pathway, although some further studies are required to explore the detailed mechanisms about ESD-mediated anticancer effect.

3.5. *ESD arrested cell cycle at G2/M phase and induced apoptosis in HeLa cancer cell*

To further investigate the ESD-mediated tumor cell growth inhibition, we examined the DNA content and cell cycle distribution of ESD-treated cells by flow cytometry. The results showed that ESD could induce apoptosis and cell cycle arrest at G2/M phase of HeLa cell in a dose-dependent manner (Fig. 6A). In detail, cell population in G_1 phase decreased from 58.40% in non-treated control cells to 26.12 and 17.15% in cells treated with 10 and 20 μM of ESD, respectively; and G2/M phase cells increased from 20.20% (control) to 63.50% (20 μM). The apoptosis induced by ESD was examined by PARP cleavage assay [14] and caspase activation based investigation [25]. As shown in Fig. 6B, ESD could dose-dependently cleave the PARP protein, and the bands of caspase 3 and caspase 9 were decreased at 24 h with the addition of ESD in HeLa cell in a dose-dependent manner. These results thereby suggested that ESD could significantly induce apoptosis in HeLa cell.

4. Discussion

Deoxyelephantopin (ESD) is a sesquiterpene lactone isolated from *Elephantopus carolinianus* Willd plant that demonstrates anti-tumor, anti-inflammatory and invasion-inhibiting activities [26]. Recently, Ichikawa et al. [14] reported that ESD could inhibit both the NF-κB activation and the NF-κB-regulated gene expression by repressing IKK, which phosphorylates and degrades I-κB subunit and induces nuclear translocation of p65 and p50 subunits. However, the direct target for ESD still remains unclear. In the present study, PPARγ was determined as the potential target for ESD as investigated by SPR and transactivation assays. Molecular docking with site-directed mutagenesis investigation further suggested that different from the typical full agonist rosiglitazone, ESD adopts a distinct binding mode against PPARγ, in which ESD failed to interact with residues His323 and His449 where rosiglitazone

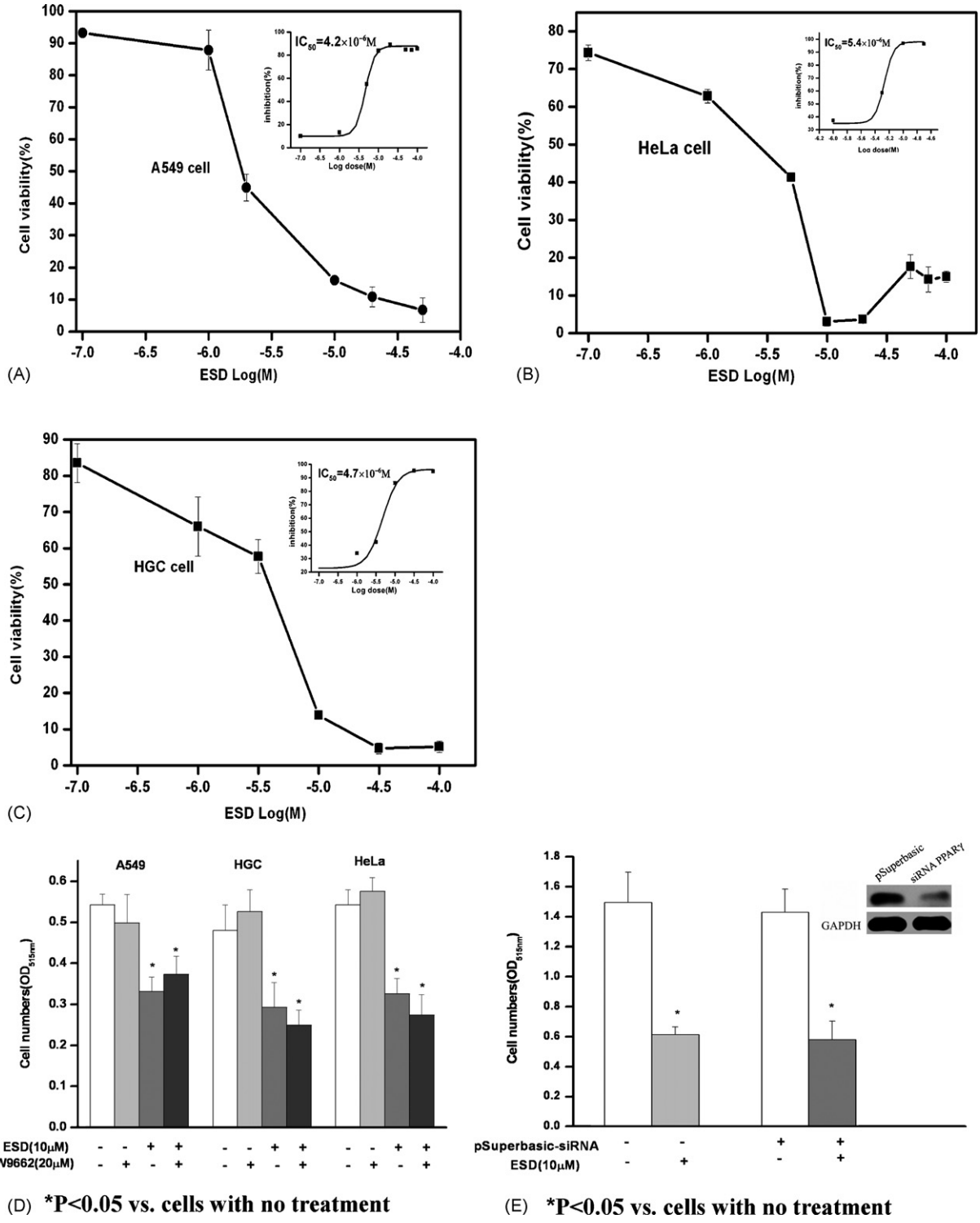

Fig. 5 – ESD inhibits proliferation of different cancer cells through PPARγ-independent pathway.
(A) A549, (B) HeLa and (C) HGC cells were seeded into a 96-well plate with 5000 cells per well and incubated at 37 °C for 24 h and then the cells were treated with increasing concentrations of ESD for another 72 h. Inset: the fitting IC_{50} value of ESD against each cancer cell. (D) A549, HeLa and HGC cells were pretreated with or without GW9662 (20 μM) for 45 min before addition of ESD (10 μM) for another 24 h. *$p < 0.05$ vs. cells with no treatment. (E) HGC cell was transfected with PPARγ siRNA and pSuperbasic (control group) as indicated in "Section 2" and then the cells were treated with or without ESD (10 μM) for 24 h. Inset: the PPARγ protein level was decreased by transfected siRNA PPARγ in HGC cell. The cell numbers were measured by SRB assay as indicated in "Section 2". Each point was performed in triplicate, and the figure is a representative of four independent experiments. *$p < 0.05$ vs. cells with no treatment.

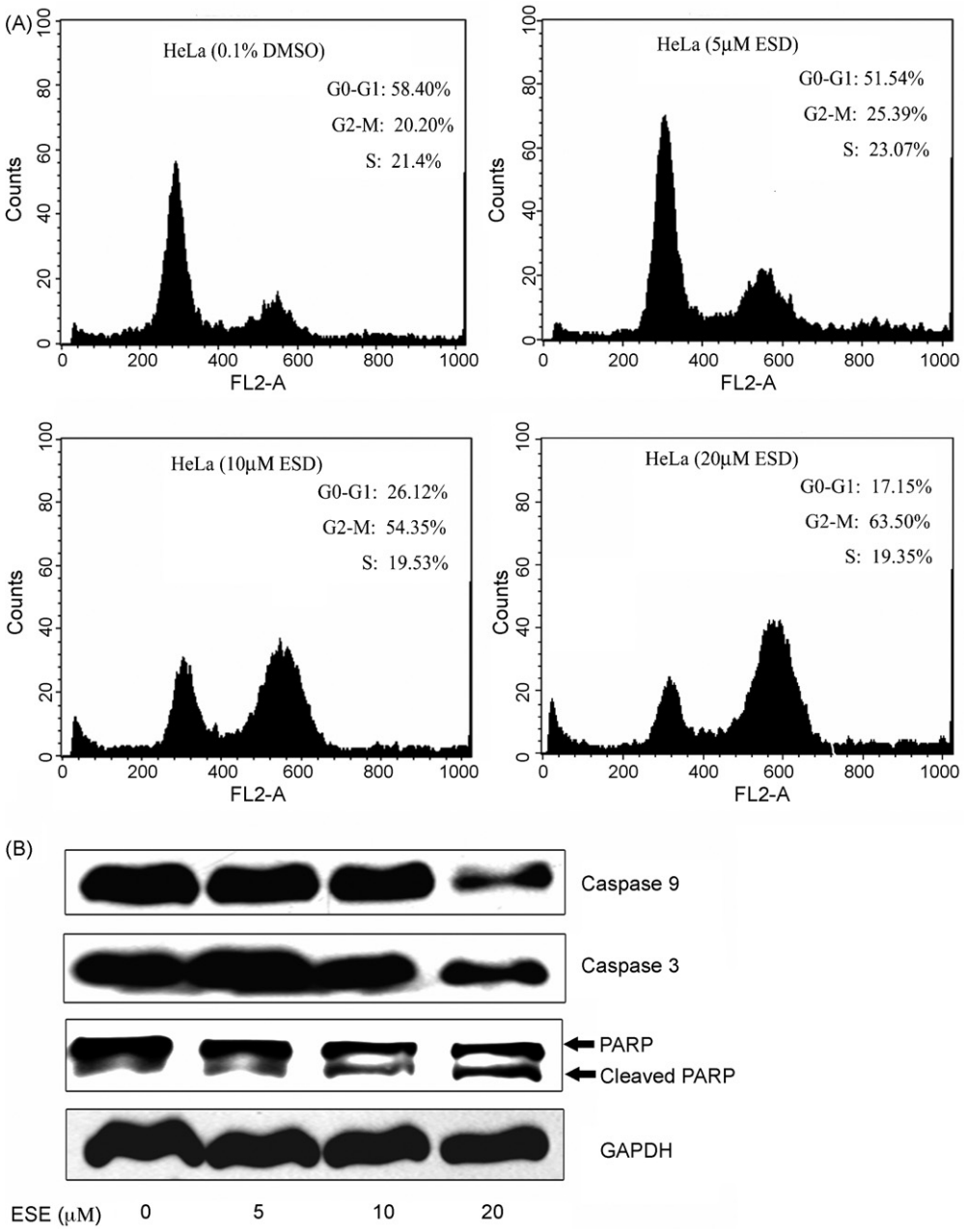

Fig. 6 – Induction of cell cycle arrest and apoptosis in HeLa cancer cell by ESD. (A) Cells were incubated with increasing concentrations of ESD for 24 h, and then collected, fixed, stained with propidium iodide and analyzed for DNA content by flow cytometry. Percentages of cells in different phases of cell cycle: G1, G2, S and sub-G1 are indicated in each panel. The X-axis means DNA content, and the figure is a representative of four independent experiments. (B) HeLa cells were incubated with 10 μM ESD for 24 h. The cells were harvested and lysed in protein loading dye, subjected to SDS-PAGE, and blotted with an anti-PARP, anti-caspase 3, anti-caspase 9 antibodies.

usually binds [24]. Moreover, the results that ESD located at the entrance of the binding pocket of PPARγ with only one hydrogen bond to Ser342 and failed to directly interact with AF-2 implicated that such ESD binding to PPARγ is more of partial agonistic feature [27], similar to the binding cases for the partial PPARγ agonists PA-082 [27], 2-BABA [28], nTZDpa [29] and AGP [30], in which the hydrogen bonding to AF-2 was not essential.

PPARγ is regarded as a potential target for the discovery of anti-cancer agents and various of PPARγ ligands have been discovered to exhibit anti-proliferation activities against a wide variety of cancer cells, although the detailed mechanisms still remain unclear [31]. Some studies demonstrated that PPARγ activation could repress proinflammatory transcription factors such as AP-1, STAT and NF-κB by PPARγ-dependent pathway [32]. For this respect, three models for the cross-talk

between PPAR and NF-κB have been reported: (1) PPAR might inhibit NF-κB activation by inducing IκB synthesis [33]; (2) PPARγ might compete with NF-κB for essential coactivators thus inhibiting NF-κB activation through transrepression mechanisms [34,35] and (3) PPARγ might be targeted to nuclear receptor corepressor (NCoR)–histone deacetylase-3 (HDAC3) complexes on NF-κB gene promoters by SUMOylation in its ligand-binding domain [36]. However, several previous reports indicated that for some types of the PPARγ ligands, their anti-cancer effects might be independent of PPARγ activation. For example, 15d-PGJ_2 is a PPARγ agonist and shows inhibition activity against cancer cell proliferation without subjecting to PPARγ activation. It is reported that 15d-PGJ_2 could repress NF-κB-related gene expression through covalent modification of critical cysteine residues in I-κB kinase and prevented the nuclear translocation of NF-κB [37]. In addition, some studies revealed that PPARγ classical agonists TZDs (e.g. troglitazone) exhibit anti-cancer effects via a PPARγ-independent pathway and some non-PPARγ targets such as extracellular signal-regulated kinases, c-Jun N-terminal protein kinase, p38 and Bcl-2 members have been implicated [38,39]. In fact, the detailed PPARγ-dependent or -independent anti-cancer mechanism study for PPARγ ligands has become an alluring project [31]. In our work, we demonstrated that ESD is a PPARγ partial agonist and exhibits anti-cancer effect possibly through a PPARγ-independent pathway as investigated by siRNA and PPARγ specific-inhibition based assays. As indicated above, there seems to be a big complexity concerning the anti-cancer characters for the PPARγ ligands. By an unclear anti-cancer mechanism, ESD might function in a mode similar to the published PPARγ partial agonist CDDO [7,40,41].

As has been investigated in this work, ESD exhibits potent anti-proliferation effects on several tumor cell lines, and the previous report indicated that ESD could repress NF-κB activation by inhibiting IκBα kinase (IKK) to disrupt the phosphorylation and degradation of the IκBα subunit and subsequent nuclear translocation of p65 subunit [14]. Therefore, with all these facts in mind, we proposed that ESD might take a PPARγ–NF–κB mediated pathway for performing its anti-cancer activity, although further studies are required for confirmation of this ESD-mediated PPARγ–IκB–NF–κB network.

Recently, the studies have indicated that PPARγ activation correlated with the inhibition against the proliferation of cancer cells by inducing cell cycle arrest at G0/G1 phase, although the exact mechanism of PPARγ in controlling cell cycle remains incompletely understood [42]. In our work, we also investigated that rosiglitazone induced cell cycle arrest at G0/G1 phase in HeLa cell (Supplementary Fig. S4), compared with the arresting at G2/M phase by ESD. Interestingly, Fajas et al. [43] recently reported that PPARγ activation caused cell cycle arrest at G0/G1 phase in the presence of retinoblastoma protein (RB), whereas resulted in accumulating in G2/M in the absence of RB. Moreover, PPARγ ligands 15d-PGJ2 and troglitazone induced cholangiocarcinoma cell cycle arrest at G2/M through p53-dependent GADD45 and $p21^{WAF1/Cip1}$ pathway [44]. Similar to the case for the known PPARγ partial agonist CDDO [7], ESD could also inhibit cell proliferation by inducing G2/M cell cycle arrest in cancer cells. Therefore, our work might possibly provide further insight into the cell cycle analysis caused by the partial agonist induced PPARγ activation in tumor cells.

Together with the previously published results [14], we expected that our work might help facilitate in providing novel insight into the underlying anti-tumor mechanism for ESD. The multifunctional characters of ESD, including PPARγ activation, NF-κB inhibition, anti-proliferation effect and apoptosis induction in tumor cells have made ESD a potential lead compound for further research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.11.021.

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