Original Research

Eriodictyol mediated selective targeting of the TNFR1/FADD/TRADD axis in cancer cells induces apoptosis and inhibits tumor progression and metastasis

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ABSTRACT

While the anti-inflammatory activities of Eriodictyol, a plant-derived flavonoid is well-known, reports on its anti-cancer efficacy and selective cytotoxicity in cancer cells are still emerging. However, little is known regarding its mechanism of selective anti-cancer activities. Here, we show the mechanism of selective cytotoxicity of Eriodictyol towards cancer cells compared to normal cells. Investigation reveals that Eriodictyol significantly upregulates TNFR1 expression in tumor cells (HeLa and SK-RC-45) while sparing the normal cells (HEK, NKE and WI-38), which display negligible TNFR1 expression, irrespective of the absence or presence of Eriodictyol. Further investigation of the molecular events reveal that Eriodictyol induces apoptosis through expression of the pro-apoptotic DISC components leading to activation of the caspase cascade. In addition, CRISPR-Cas9 mediated knockout of TNFR1 completely blocks apoptosis in HeLa cells in response to Eriodictyol, confirming that Eriodictyol induced cancer cell apoptosis is indeed TNFR1-dependent. Finally, in vivo data demonstrates that Eriodictyol not only impedes tumor growth and progression, but also inhibits metastasis in mice implanted with 4T1 breast cancer cells. Thus, our study has identified Eriodictyol as a compound with high selectivity towards cancer cells through TNFR1 and suggests that it can be further explored for its prospect in cancer therapeutics.

Introduction

Chemotherapy alone is seldom a feasible option owing to the development of chemoresistance, thereby, allowing tumor cells to escape drug action and resulting in cancer cell survival and treatment failure [1–3]. Hence, the need is to identify potential anti-cancer compounds, preferably from natural products, with novel modes of action that can selectively target the apoptotic machinery of the tumor cells without affecting the normal cells.

Some natural plant-based medications have limitations, such as adverse drug responses, toxicity, and safety, which restrict their therapeutic potential and necessitate improvement through medicinal chemistry [4–6]. Despite extensive testing of a wide range of natural plant-derived medicines, only a handful (such as Paclitaxel, Vinblastine, ABBREVIATIONS: DISC, Death Inducing Signaling Complex; TNFR1, Tumor Necrosis Factor Receptor 1; FADD, FAS-Associated Death Domain Protein; TRADD, Tumor Necrosis Factor Receptor type 1-Associated DEATH Domain protein; CRISPR-Cas9, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9; RSK-2, Ribosomal S6 Kinase 2; ATF1, Activating Transcription Factor 1; NFκB, Nuclear Factor Kappa-light-chain-enhancer of activated B cells; TNF-α, Tumor Necrosis Factor-alpha(α); PARP1, Poly [ADP-Ribose] Polymerase 1; DMSO, Dimethylsulfoxide; FACS, Fluorescence Activated Cell Sorting; PE, Paired-End; PI, Propidium Iodide; PBS, Phosphate Buffer Saline; SDS-PAGE, Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis; TBST, Tris Buffered Saline (Tween 20); PVDF, Polyvinylidene fluoride; ECL, Enhanced Chemiluminescence; OD, Optical Density; DTT, Dithiothreitol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IC50, Half-maximal Inhibitory Concentration; CDK1, Cyclin-Dependent Kinase 1; IPA, Ingenuity Pathway Analysis; rh TNF, Recombinant human Tumor Necrosis Factor. * Corresponding author.
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Therefore, we have highlighted Eriodictyol as a potent compound specifically targeting cancer cells of the cervical, breast, colon and renal origin and identified the mechanism behind its selective cytotoxicity in cancer cells compared to normal cells. Hence, this study supports the potentiality of Eriodictyol to be investigated as a promising chemotherapeutic agent.

Materials and methods

Reagents

Eriodictyol (#89061) was purchased from Merck, India. Primers were purchased from IDT, USA. Primary antibodies TRADD (#3994), FADD (#2782), TNFR1 (#3736), Bax (#3994), Survivin (#2808), Claspin (#2800), γH2AX (#80312), p21 (#2947), Caspase 7 (#9494), Caspase 8 (#9746), HSP90 (#4874), Cdc25A (#3652) and PARP1 (#9532) were obtained from Cell Signaling Technology (CST), USA. Bcl2 (sc-7382) was bought from Santa Cruz Biotechnologies (USA). Housekeeping genes β-actin (BB-AB0024) and GAPDH (BB-AB0060) were obtained from Biobharati Life Sciences Pvt. Ltd. (India), Cdc2 (ab 18), phospho-Cdc2 (Tyr15) (ab17549), Cyclin B1 (ab20535) primary antibodies and colorimetric assay kit of caspase 3 (ab39401) were obtained from Abcam, Cambridge, UK. Santa Cruz Biotechnologies provided the Z-VAD-FMK (sc3067). Gibco, USA provided FBS (#16000044), L-glutamine, gentamicin, sodium pyruvate and MEM non-essential amino acids.

Cell culture and maintenance

HeLa (Human Cervical cancer), HCT116 (Human Colon cancer) and WI-38 (Normal lung fibroblast) cell lines were originally obtained from ATCC (USA). Additionally, human cell lines, NKE (Normal kidney epithelial) and SK-RC-45 (Renal cancer cells), as well as murine tumor cell lines, 4T1 (Breast cancer), CT-26 (Colon cancer) and Renca (Renal cancer) were a kind gift from Dr. James H. Finke (Cleveland Clinic, USA) and Dr. Gerd Ritter (Ludwig Institute of Cancer Research, USA) respectively. HEK (Human embryonic kidney) and MCF-7 (Human Breast cancer) cell lines was obtained from NCCS, Pune, India. The cell lines described above were grown in either RPMI 1640 or DMEM with 10% FBS and other supplements at 37 °C in presence of 5% CO₂.

MITT cell proliferation assay

MITT assay was performed to determine the effect of Eriodictyol on cellular proliferation of normal and cancer cell lines. Briefly, cells were grown in 48 well plate formats in presence of Eriodictyol (0–200 µM) for 72 h. Phenol red-free RPMI without serum containing MTT (0.5 mg/mL; 200 µL per well) were added to each well and incubated at 37 °C for 3 h in a humified incubator containing 5% CO₂. The purple-colored formazan crystals formed by cells were dissolved in DMSO and absorbance was measured at 570 nm in a microplate reader. The selectivity index for human cancer cell lines SK-RC-45, HeLa, HCT-116 and MCF-7 was calculated from the ratio of their respective IC₅₀ values and that of normal cell lines (WI-38 and NKE).

Clonogenic survival assay

For clonogenicity assay, cells were plated at very low density (200 cells/well in 6 well plates), grown for either 8/10 days, fixed, and stained with 3.7% formaldehyde and 0.05% crystal violet. The assay provides a measure of the replicative capacity of the cells, which is dependent on the ability of the cells to form clones from a single cell. Images of wells containing cells were captured using Gel Doc XR+ (Bio-Rad). The method of quantification of the clones is mentioned in the respective legend.

Cell cycle profiling assay

For this experiment, HCT-116 cells were treated with increasing concentrations (100 µM, 150 µM, 200 µM) of Eriodictyol for 24 h, while HeLa cells were treated with 50 µM, 100 µM, 200 µM of Eriodictyol for 24 h. Post-treatment, cells were harvested into a single cell suspension and fixed by incubating the cells overnight at -20 °C with 75% ethanol. Cells were centrifuged and resuspended in 1 × PBS for 2 h followed by RNase A (20 µM) treatment for 2 h at 37 °C. Finally, cells were incubated with Propidium Iodide (PI) for 15 min at room temperature. Flow cytometric analysis was immediately performed using FACSuite software.

Annexin-V-FITC/PI staining

Briefly, post-treatment with Eriodictyol, 2 × 10⁵ cells were washed with ice-cold 1X PBS and resuspended in 100 µL of 1X binding buffer. Cells were incubated with 5 µL of annexin-V-FITC followed by 5 µL of PI for 15 min at room temperature in a dark place as per the manufacturer’s protocol. Finally, the percentage of stained cells was measured in the FACSc-Verce instrument (BD) and analyzed using FACSuite software.

RNA sequencing data analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified by DNase treatment (ThermoFisher Scientific). The quality of the RNA samples was checked on Agilent TapeStation and quantified by Qubit fluorometer. The PE Illumina libraries were loaded onto NovaSeq6000 for cluster generation and sequencing. The trimmed reads were aligned with Human GENCODE hg38 reference genome using Hisat2
2.1.0. Following the sorting of Bam files with Samtools v1.19, transcripts assembly and differential gene analysis were performed with Cufflinks v2.2.2.1 and Cuffdiff v2.2.1, respectively, and differentially expressed genes with a fold change greater than equal to 2 and p value cut off of less than or equal to 0.05 were filtered out. Finally, gene enrichment and pathway analysis were performed with IPA (Ingenuity Pathway Analysis).

Western immunoblotting

Briefly, cell lysates were prepared from HeLa cells post-treatment with Eriodictyol (150 µM) using RIPA buffer. For immunoblotting, 30–50 µg proteins were resolved on 10–15% SDS-PAGE, transferred onto PVDF/nitrocellulose membrane (Millipore, USA), blocked with 5% BSA dissolved in 1X TBST, and subsequently probed with respective primary antibodies and secondary antibodies. Finally, blots were developed onto X-Ray films by the ECL method or by using gel imaging system (ChemiDoc MP, Bio-Rad and iBright Imager, Invitrogen) [13].

Caspease-3/7 activation assay

The assay was based on the ability of the enzyme(s) to detect and cleave the sequence DEVD from the chromophoric substrate DEVD-pNA. Caspase-3/7-dependent sequence specific cleavage of DEVD-pNA to release p-NA (p-nitro-aniline) from the substrate and its colorimetric detection enables quantitative measurement of the activity of Caspase-3/7 in the lysates. For this, cell lysates were prepared from HeLa using RIPA buffer following treatment with Eriodictyol. An aliquot of 150 µg of protein was used for each condition. DTT and DEVD-pNA substrate were added according to the manufacturer’s (Abcam #ab304011) instructions. Samples were mixed well and incubated at 37°C for 90 min and finally, OD was measured at 405 nm in Multiskan GO microplate reader (ThermoFisher Scientific) [15].

Design, construction and validation of TNFR1 CRISPR

Guide oligos (20-mers) were designed from TNFR1 exon 2 sites using http://crispr.mit.edu (Supplementary figure S1A), on the basis of high "on-target score" and low "off-target score" in the whole exome. Guide oligo was then cloned into Cas9 expression vector (psPcCas9(BB)-2A-Puro) (Supplementary figure S1 B) having a puromycin selection cassette, sequence verified and TNFR1-CRISPR plasmid was transfected into HeLa cells using lipofectamine LTX reagent (Invitrogen) to check the functionality of the CRISPR plasmdis within the cells. Following initial selection with puromycin (1 mg/ml) over the week, genomic DNA was isolated from transfected cells and a mismatch heteroduplex sensing T7E1 assay was performed [17]. Next, clonal expansion from single cells was performed followed by immunoblotting of clonally expanded cells to screen for stable TNFR1-CRISPR knockout cells.

Real-time PCR and RNA isolation

Total RNA was extracted using TRIzol reagent (Invitrogen). 1 µg of RNA was extracted from HeLa cells treated with Eriodictyol along with DMSO-treated control. Verso cDNA synthesis kit (ThermoFisher Scientific) was used to convert RNA to cDNA. Real-Time PCR was performed in triplicate with a diluted stock of cDNA (1:6) mixed with SYBR green on Real Time PCR system 7500 Fast (Applied Biosystems) or QuantStudio 3 Real Time PCR system. Every mRNA quantification data were normalized to GAPDH and expressed as the fold differences of target mRNA levels in triplicate with a diluted stock of cDNA (1:6) mixed with SYBR green on Real Time PCR system 7500 Fast (Applied Biosystems) or QuantStudio 3 Real Time PCR system. Every mRNA quantification data were normalized to GAPDH and expressed as the fold differences of target mRNA levels.

4TI breast tumor model

Female Balb/c mice aged 6–8 weeks were obtained from the Center for Translational Animal Research (CTAR) at the Bose Institute in Kolkata, India. The protocol was approved by the Bose Institute Animal Ethics Committee (Approval No. IAEC/BI/118/2018, dated 20/12/2018) and all animal experiments were performed in accordance with the institutional guidelines and in compliance with the CPCSEA regulations (based on NIH guidelines) based on the recommendations of the institutional animal ethics committee. 4TI cells (1 × 105 cells/animal) were subcutaneously implanted into the mammary fat pad of Balb/c mice for the in vivo tumorigenic experiment. Following the formation of a palpable tumor, animals were randomly divided into two groups: vehicle control (5% DMSO) and Eriodictyol-treated (60 µg/kg), each consisting of at least five animals, and were subjected to intraperitoneal injections beginning around 10 days after tumor formation and lasting until 22 days (5 doses, 72 h interval). On every third day, the volume of the tumor was measured with vernier calipers using the equation Tumor volume = 0.5 × a × b², where “a” and “b” indicate major and minor axis, respectively.

4TI tumor metastasis in secondary organs

4TI tumor cells (1 × 105) were injected intravenously through the tail vein in syngeneic Balb/c mice. Post-inoculation, mice were randomly divided into three groups and administered with either 5% DMSO or two different doses of Eriodictyol, thrice a week. 3-4 weeks post-injection of tumor cells, animals were sacrificed and scrutinized for micro-metastatic nodule formation in the lungs, which is indicative of metastasis, and subjected to the counting of the number of micro-metastatic nodules as well as tissue staining of the lung cross-sections with hematoxylin-eosin (H&E).

Statistical analysis

Statistical analysis was performed by Student’s t-test using GraphPad Prism 5.0 software. Statistical comparisons between groups were determined by a one-tailed or two-tailed Student’s t test. The data are represented as mean ± SEM of at least three independent experiments, unless mentioned otherwise. The p values were used to ascertain if the results are statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant). The statistical analysis of each experiment is detailed in the respective legends.

Results

Eriodictyol induces selective cytotoxicity to cancer cell lines

MTT cell viability assay indicates that Eriodictyol displays high toxicity towards human cancer cells SK-RC-45 (IC50=53.75 µM), HeLa (IC50=107.5 µM), HCT-116 (IC50=105 µM) and MCF-7 (IC50=75 µM) as well as murine cancer cells, 4TI (IC50=75 µM), CT-26 (IC50=47.5 µM) and Renca (IC50=145 µM) dose-dependently (Figs. 1A-D). Interestingly, it is negligibly toxic towards normal human lines, NKE and WI-38 as indicated by IC50>200 µM (Figs. 1E, 1F). The selectivity index (SI) measures a compound’s cytotoxicity against cancer cells relative to non-tumor cells. The higher a compound’s SI, the more selective it is. The SI of Eriodictyol is calculated as the ratio of IC50 on normal cell line (NKE and WI-38) to the IC50 on cancer cell line (SK-RC-45 SI=3.72, HeLa SI=1.86, HCT116 SI=1.9, MCF-7 SI=2.66). The SI value of more than 1.5 has been considered as high selectivity. Therefore, this data not only demonstrates the selective cytotoxicity of Eriodictyol against different cancer cell lines over normal cells but also helps us in selecting the mouse tumor cell line for in vivo studies.
Fig. 1. Eriodictyol elicits selective cytotoxicity against several cancer cell lines. A, C, E. Line graphs showing in vitro cytotoxicity measured through MTT assay in presence of Eriodictyol (0-200 µM) at 72 h in human cancer (SK-RC-45, HeLa, HCT-116, MCF-7), mouse cancer (4T1, CT-26, Renca) and normal human (WI-38 and NKE) cell lines. The results are summarized from at least three independent experiments. B. Table depicting IC_{50} values and SI of human cancer cells. Selectivity index = mean IC_{50} ratio (normal cell line/cancer cell line). D, F. Displays the IC_{50} values of Eriodictyol in mouse cancer and normal human cell lines respectively. G, I. Pictorial representation of plates and H, J. Column graph showing dose-dependent inhibition of clonogenicity in SK-RC-45 (quantification of cell viability based on densitometry with image analysis by Bio-Rad ChemiDoc XRS) and HeLa cell lines (quantified by manual cell count) respectively, in response to Eriodictyol treatment. The results are shown as mean ± SEM of three independent experiments. Student’s t-test were used for analysis (two-tailed; *p < 0.05, **p < 0.01, ***p < 0.001 vs non-treated, ns not significant).
Eriodictyol blocks clonogenicity of cancer cells

The ability of a single cell to form clones, as represented by clonogenicity, is a defining characteristic of cancer cells. Eriodictyol inhibits clonogenicity of SK-RC-45 cells at concentrations as low as 10 \( \mu \text{M} \) with a significant reduction in colony formation at 25 \( \mu \text{M} \) of Eriodictyol (Figs. 1G, H). Moreover, it shows better efficacy in HeLa cells with inhibition of clonogenicity starting from 2.5 \( \mu \text{M} \) with an almost complete regression of colony formation at 25 \( \mu \text{M} \) of Eriodictyol (Figs. 1I, J). It is clearly evident from the data, that while Eriodictyol significantly inhibits colony growth at a concentration as low as 2.5 \( \mu \text{M} \) in the case of HeLa cells, and 25\( \mu \text{M} \) in SK-RC-45 cells (Figs. 1G-J), the concentration of Eriodictyol that causes \( \sim 40-50\% \) cells to undergo apoptosis at least in the case of HeLa and MCF-7 cells is 125 \( \mu \text{M} \) (Figs. 2A,B), which is evidently several folds higher than that required to inhibit significant clonogenicity. Therefore, we conclude that Eriodictyol effectively inhibits the clonogenicity of cancer cells.

Eriodictyol induces apoptosis in human and murine cancer cells

Apoptosis is characterized by the translocation of the phosphatidylserine (PS) from the cytosol to the cell membrane, which is experimentally detected by binding of annexin V-FITC to PS. Our flow cytometry study reveals that Eriodictyol treatment for 48 hours causes dose-dependent activation of apoptosis in HeLa and MCF-7 cells, as evidenced by a dose-dependent increase in the percentage of annexin-V +ve cell populations (Figs. 2A, B), while it caused only moderate, yet significant induction of apoptosis in HCT-116 cells (Fig. 2C). Similarly, Eriodictyol also induces apoptosis in mouse tumor cells, 4T1 and CT-26 (Figs. 2D, E), as indicated by a dose-dependent increase in the percentage of annexin-V +ve cells. Interestingly, a comparative study of the apoptosis induction between cancer cell lines, HeLa vs normal cell lines, WI-38 and NKE confirms that Eriodictyol induces a significantly higher level of apoptosis in the cancer cell line, HeLa as compared to both the normal cells, WI38 and NKE. However, while Eriodictyol elicits only a negligible level of apoptosis in WI-38 cells, NKE cells display only a moderate level of apoptosis in response to Eriodictyol treatment, and that too from 125 \( \mu \text{M} \) (Fig. 3). Collectively, these results confirm that the selective cytotoxicity of Eriodictyol not only resides in its ability to inhibit cancer cell proliferation but also, in inducing differential apoptosis in different cancer cell lines.

Fig. 2. Eriodictyol treatment induces apoptosis in human and mouse cancer cell lines. Flow cytometric density plots demonstrating a dose-dependent increase in the annexin V-FITC/PI-positive population of (A) HeLa, (B) MCF-7, (C) HCT-116, (D) 4T1, and (E) CT-26 cells in response to Eriodictyol treatment. Post-treatment with Eriodictyol (0–150 \( \mu \text{M} \)) for 48 h, cells were stained with annexin V-FITC/PI, apoptosis was measured in BD FACS Verse instrument and analyzed in BD FACSuite™. Data represent at least three independent experiments. Column graphs represent concentration-dependent induction of apoptosis in the above-mentioned cell types and experimental conditions. The data are represented as Mean \( \pm \) SEM of three independent experiments and analyzed by Student’s t-test (two-tailed); \( *p < 0.05, **p < 0.01, ***p < 0.001, \text{ns not significant} \). The total apoptosis population was calculated by combining the quadrants UL+UR+LR.
Eriodictyol arrests cell cycle progression at the G2/M phase

Flow cytometric determination of the cell cycle distribution of PI-stained cervical and colon cancer cells indicates dose-dependent accumulation of HeLa (Figs. 4A, B) and HCT-116 (Figs. 4C-E) at the G2/M phase of the cell cycle upon Eriodictyol treatment. The activation of Cdc2 (CDK1) through dephosphorylation of Cdc2 at Thr14 and Tyr15 by a group of phosphatases e.g., Cdc25 has been reported as a critical regulatory step in the progression of cells to mitosis [19]. As evident from the Western blot (Fig. 4E), Eriodictyol causes a time-dependent increase in phosphorylation of Cdc2 at Tyr15, in addition, to a decrease in expression of Cdc2, Cdc25A indicating de-activation of Cdc2, consequently leading to a delay in cell cycle progression to mitosis and holding the cells at the G2/M checkpoint.

Inhibition of Cdc2 by Eriodictyol results in the accumulation of cells in the G2 phase without mitosis, thus, resulting in the accumulation of Cyclin B1.

Eriodictyol stimulation induces differential expression of distinct gene sets implicated in proliferation, cell-cycle and apoptosis-related pathways in the HeLa cell line

To explore the extent of Eriodictyol mediated modulation of gene expression and associated biological processes, transcriptome sequencing is performed in Eriodictyol-treated versus non-treated cells at both early (6 h) and late (24 h) time points in HeLa cells. The primary analysis of the data on the Differentially Regulated Gene sets (DRGs) show distinctly different clustering of gene sets as evidenced from the heat map (Fig. 5A) in Eriodictyol treated versus non-treated cells. The transcripts with fold change (FC) ≥2 with p-value ≤0.05 are selected for our analysis. The volcano plot depicts a marked increase in the number of upregulated (apoptosis inducers and tumor suppressors) and downregulated (metastasis inducers and cell cycle regulators) genes (Fig. 5B). Gene enrichment and pathway analysis have been performed with IPA (Ingenuity Pathway Analysis) and the results are grouped into three clusters (metastasis, apoptosis, and cell cycle) and represented as Venn diagram (Fig. 5C). Moreover, we have established a heat map displaying common tumor-related genes mostly affected by Eriodictyol (Fig. 5D), which displays a distinctly different gene expression profiling in the Eriodictyol treated versus non-treated cells. Data clearly shows distinct upregulation of apoptosis inducers and tumor suppressors and a significant decrease in oncogenes and cell cycle regulators in response to Eriodictyol. Finally, significant modulation of key biological processes in response to Eriodictyol treatment, many of which are closely related to or involved in apoptosis, metastasis and cell cycle regulation are represented as bar diagram (Fig. 5E). The results of canonical pathway analysis indicate that Eriodictyol may be involved in cell cycle regulation, specifically at the G2/M phase. In addition, pathway analysis
Fig. 4. Eriodictyol disrupts cell cycle progression by causing G2/M arrest. A. Histogram represents a dose-dependent increase in the G2/M phase population of Eriodictyol treated HeLa cells compared to untreated control cells for 24 h. B. Bar graph represents the percentage of HeLa cells at various phases of the cell cycle in response to Eriodictyol treatment. C. Histogram showing a dose-dependent increase in G2/M population and a corresponding decrease in G1 phase population in HCT-116 at 24 h post-treatment of Eriodictyol. D. Column graph depicting % of HCT-116 cells residing in various phases of the cell cycle in response to corresponding concentrations (0–200 µM) of Eriodictyol. Data represents Mean ± SEM of three independent experiments and Student’s t-test (two-tailed; *p < 0.05, **p < 0.01, ***p < 0.001, ns not significant) was used for significance analysis. E. Western Immunoblots showing G2/M phase Cyclins and CDK protein expression at various time points (0–72 h) in response to Eriodictyol treatment. HSP90 was used as the loading control.
reflects several pathways that are involved in the regulation of Cell Cycle, DNA-damage as well as Apoptotic responses, some of which are represented in Fig. 5 F. Interestingly, TNFR1 is one of the genes that is not only significantly upregulated but also commonly involved in several of these pathways. Taken together, Eriodictyol causes differential regulation of gene expression that translates into the induction of apoptosis, cell cycle arrest and cytotoxicity to the tumor cells.

Eriodictyol disrupts pro-apoptotic/anti-apoptotic signal balance in cancer cells

Investigation of the detailed molecular events indicate the involvement of the DISC complex in Eriodictyol mediated apoptosis of HeLa cells, as evident from significant time-dependent upregulation in expression of the pro-apoptotic DISC components, i.e., TNFR1, FADD and TRADD (Fig. 6A).

In addition, Eriodictyol causes significant time-dependent increase of pro-apoptotic Bax, with a dramatic time-dependent decrease in anti-apoptotic Bcl2 and Survivin (Fig. 6B), thereby tipping the balance towards a pro-apoptotic outcome. Eriodictyol also causes time-dependent downregulation in the expression of the key anti-apoptotic and DNA-damage response protein, p21 in HeLa cells (Fig. 6C).

Furthermore, Eriodictyol treatment results in a time-dependent increase in the expression of the DNA fragmentation marker, γ-H2A.X, indicating that Eriodictyol may have a DNA damaging effect in cancer cells, perhaps leading to the activation of downstream apoptotic processes (Fig. 6C). Eriodictyol-mediated apoptotic cascade involves caspases as evident by a time-dependent reduction in pro-caspase 7 expression and an increase in cleaved caspase 7, activation of caspase 8, as well as a decrease in full-length 116 kDa PARP1 levels and the presence of the cleaved 89 kDa form (c-PARP1) (Fig. 6D). As shown in Fig. 6E, the caspase activity assay also confirms activation of caspase 3/7 in HeLa cells at later time points (24, 48 and 72 h). Pretreatment of HeLa cells with 20 µM inhibitor of PAN-caspase (Z-VAD-FMK) provide a

Fig. 5. Eriodictyol treatment induces differential expression of distinct gene sets implicated in apoptosis, metastasis, cell-cycle regulatory genes, and associated pathways in HeLa cells. A. A heat map showing differential regulation of global genes in response to Eriodictyol treatment in both 6 h (E6_1, E6_2) and 24 h (E24_1, E24_2) in comparison to untreated control (C_1, C_2). B. Volcano plot depicting the differentially regulated global genes at 6 h post treatment of Eriodictyol. C. Venn diagram analysis exhibiting total number of apoptosis, metastasis and cell cycle regulatory genes at 6 h Eriodictyol treatment. D. Heat map displaying a screened set of genes highly affected by Eriodictyol at 6 h through IPA analysis. E. Functional analysis represented as bar plot C vs E6. F. Ingenuity Pathway Analysis. The color intensity of the nodes shows the degree of enrichment of this analysis. The enrich-factor is defined as the ratio of the differential genes in the entire genome. The dot size represents the count of genes in a pathway. C_1- Untreated sample 1, C_2- Untreated sample 2, E6_1 - Treated sample 6 h sample 1, E6_2 - Treated sample 6 h sample 2, E24_1 - Treated sample 24 h sample 1, E24_2 - Treated sample 24 h sample 2.
moderate (~31% inhibition), yet significant protection against Eriodictyol-mediated apoptosis (Figs. 6 E, F), confirming that Eriodictyol mediated tumor cell apoptosis is at least partially caspases-dependent. Thus, collectively we confirm that Eriodictyol upregulates the pro-apoptotic proteomes and causes a significant reduction in survival proteins, thereby, culminating in a pro-apoptotic outcome in cancer cells.

TNFR1 is the potential target of Eriodictyol and the primary basis behind its selective cytotoxicity

Our preliminary data indicate that Eriodictyol treatment remarkably increases the mRNA levels of both TNF-α and TNFR1 in a time-dependent manner (Figs. 7A, B). Further, both the mRNA and protein levels of TNFR1 are elevated in HeLa cells, post-Eriodictyol treatment at 150 µM (Figs. 7 B, C). Multiple studies have reported that TNFR1 is involved in the extrinsic pathway of apoptosis [20]. To further elucidate the relationship between the selectivity of Eriodictyol towards cancer cells over normal cells, the basal expression level of TNFR1 in non-cancerous cells (HEK, NKE and WI-38) versus cancer (SK-RC-45 and HeLa) cells. Western blot results clearly show that the basal level of TNFR1 protein expression in cancer cells is significantly higher than that in normal cells (Fig. 7D), thereby indicating that lack of expression of TNFR1 receptor in normal cells may be the reason for the observed selective activity of Eriodictyol against the cancer cells.

To further confirm, whether Eriodictyol selectively induces TNFR1 expression, the relative expression of Eriodictyol induced TNFR1 was checked in both cancers as well as normal cells. As shown in Fig. 7E, 24 h and 48 h exposure to Eriodictyol results in a significantly elevated time-dependent induction in TNFR1 expression in cancer cells, while in the normal NKE and HEK cells, induction of TNFR1 expression with time is significantly lower compared to the cancer cells. Hence, this data supports the rationale for Eriodictyol’s selective cytotoxicity towards cancer cells. Furthermore, remarkable dose-dependent upregulation in the induction of TNFR1 expression in HeLa cells initiates at 100 µM concentration of Eriodictyol compared to negligible or no TNFR1 expression in NKE (Fig. 7F, top panel) and WI-38 cells (Fig. 7F, top panel).
bottom panel), respectively, which correlates with the respective dose-dependent apoptotic window in HeLa cells (Fig. 3C).

Finally, to confirm the direct role of TNFR1 in the regulation of Eriodictyol mediated apoptosis, we have established TNFR1-knockout cells using CRISPR-Cas9 genome editing technology (Figs. 8A–C; Supplementary figure S1). Out of the 10 clones screened for the expression levels of TNFR1, 4 clones (Fig. 8A) appear to have complete depletion of TNFR1 expression. TNFR1 KO clone#6 is used for subsequent studies. While Eriodictyol induces significant apoptosis in wild type HeLa cells (>45% annexin V/PI+ cells) at 150 μM, there is a
marked decrease in apoptosis in TNFR1 knockout clone KO-6 (~7% annexin-V/PI+ cells) as evident from the density plot and bar graph (Figs. 8 D, E), thereby confirming that Eriodictyol mediated tumor cell apoptosis is TNFR1-dependent. Therefore, we conclude that the TNFR1 signaling axis is important in the Eriodictyol-induced apoptosis of cancer cells.

Eriodictyol inhibits tumor development, progression and metastasis in immuno-competent mice

To translate the in vitro anticancer activity of Eriodictyol in vivo, a syngeneic mouse (Balb/c) tumor model has been used. In vivo findings demonstrate a substantial suppression of tumor development, as evident by a time-dependent decrease in tumor volume (Figs. 9A, B), as well as a decrease in the volume and weight of resected tumors from Eriodictyol-treated mice compared to non-treated animals (Figs. 9C, D). Eriodictyol-treated mice show no signs of toxicity as evident from the measurement of body weight of mice (Fig. 9E).

Moreover, Eriodictyol treatment results in a dose-dependent decrease in the number of lung metastatic nodules, (Figs. 9F, G) and a significant decline in the weight of the lungs in Eriodictyol–treated groups compared to the control group (Supplementary figure S2B). In summary, our in vivo study indicates that Eriodictyol reduces tumor growth and progression, as well as suppresses pulmonary lung metastasis of 4T1 cells in immune-competent Balb/c mice.

Discussion

One of the hallmarks of tumor cells is the evasion of apoptosis [21, 22]. Enhancement of the pro-apoptotic ability of the anti-cancer agents through abrogation of the survival signaling has been recognized as an effective strategy towards sensitizing the cancer cells to the chemotherapeutic drugs and preventing chemoresistance [23–25]. Owing to the increasing significance of the TNF pathway in cancer, the primary aim of the present study is to discover an effective TNF-mediated anti-cancer therapeutic with minimal toxicity to the normal cells and...
Fig. 9. Eriodictyol impedes the progression of mammary tumors and lung nodules in a syngeneic mouse model. A. Image of mice bearing tumors (indicated by red circles) before sacrifice, injected in the mammary fat pads subcutaneously with 4T1 cells, either treated with Eriodictyol (bottom row) or non-treated (top row). B. Line graph showing regression of tumor volume (time-dependent) in response to intraperitoneal injection of Eriodictyol, in Balb/c mice injected subcutaneously with 4T1 cells at the mammary fat pads, in comparison with those non-treated. C. Represents image of resected tumors from mice injected in the mammary fat pads subcutaneously with 4T1 cells, either treated with Eriodictyol (bottom row) or non-treated (top row). D. Bar graph showing significant reduction in tumor weight of the resected tumors, post-Eriodictyol treatment at 60 mg/kg body weight. E. Comparison of mice body weight in eriodictyol-treated vs non-treated. F. Photomicrographs depicting formalin-fixed whole lungs from male Balb/c mice injected with 4T1 cells (tail vein injection) with or without Eriodictyol treatment at different concentrations (0, 10, 25 mg/kg body weight). G. Graphical representation of the total number of lung nodules counted at different doses (0, 10, 25 mg/kg body weight) of Eriodictyol. The data represents two independent experiments and bar graph represents mean ± SEM (Student’s t-test (two-tailed), *p < 0.05, **p < 0.01, ***p < 0.001, ns not significant).
elucidate the comprehensive mechanism of its anti-cancer activity and selectivity towards cancer cells.

TNF family members are the multifunctional cytokines involved in a broad spectrum of biological processes, which includes cell proliferation, cell death, carcinogenesis, immune responses, etc. [26,27]. Binding of TNF ligands to TNF receptors on the cancer cells has been documented to activate the extrinsic apoptosis pathway [28,29], thereby, instigating the initiation of the clinical trials to evaluate the anti-cancer efficacy of rhTNF-α on advanced solid cancers [3,4]. Unfortunately, in contrast to the preclinical studies, the outcome of the clinical trials was disappointing, as evidenced by the decline in apoptosis triggering ability of rhTNF-α, which has been attributed to the simultaneous activation of downstream NF-κB survival pathway [30–32]. Based on these findings, it was inferred that the TNF-α mediated killing of malignant cells through the cell death-receptor pathway could be augmented upon concomitant blockade of NF-κB cascade [33,34]. Since, Eriodictyol activates the downstream signaling that inhibits NF-κB [35,36], we have examined whether inhibition of the same could activate the endogenous TNF-α mediated apoptosis pathway in cancer cells [37]. Previously, some reports have suggested the anti-cancer activity of Eriodictyol on different malignant cells as well as its selectivity towards cancer cells [10,11,38–40]. Nonetheless, the mechanism behind its selective targeting of cancer cells is not yet well-reported, thus, motivating us to understand the underlying mechanism of its selectivity towards cancer cells over normal cells and decipher the precise pathway of Eriodictyol-induced cancer cell death. Here, we have hypothesized that the ability of Eriodictyol to specifically target aberrations contributing to the tumor’s proliferative advantage, while sparing normal cells, can lead to a favorable outcome. Our research presents a global as well as differential regulation of gene expression profile in response to Eriodictyol. Among the several biological processes studied, Eriodictyol treatment demonstrated upregulation of a variety of pro-apoptotic and tumor-suppressor genes as well as induction of cell death, confirming its involvement in suppression of tumor cell proliferation. Taken together, our findings offer new possibilities to Eriodictyol’s hitherto unrecognized role and unknown mode of action in altering gene transcription as well as unfolding its previously undiscovered anti-cancer characteristics.

Interestingly, our investigation manifests that Eriodictyol-mediated TNFR1 upregulation and releasing of endogenous TNF-α from cancer cells (HeLa), collectively have the fortuitous effect of altering the functional state of the TNF-α pathway from NF-κB dependent cell survival to death-inducing complex formation. In addition, the importance of TNFRI in Eriodictyol-induced apoptosis is further supported by the exclusive expression of TNFRI on cancer cells (and not normal cells), wherein Eriodictyol is unable to induce any appreciable TNFRI expression in normal cells even after prolonged exposure. Additionally, TNFRI knockout causes significant abrogation of cancer cell apoptosis. Thus, this study uncovers the mechanism behind the selectivity of Eriodictyol-mediated cytotoxic phenomena in cancer cells over normal cells.

Further investigation of the mechanistic details reveals that Eriodictyol induces extrinsic pathways of apoptosis in cancer cells, as evident from the expression profile of hallmark apoptosis-related proteins, which is similar to several previously reported anti-cancer agents [16,41–46]. TNFR1-DISC-Casp-PARP1 is the major axis in the extrinsic apoptosis pathway and we are the first to report the involvement of this pathway in Eriodictyol-mediated selective cytotoxicity in cancer cells. The approach of selectively targeting the extrinsic route to induce apoptosis in tumor cells, such as through ligation of death receptors, is appealing for cancer therapy, since death receptors have a direct link to the cell’s death machinery [47]. Furthermore, tumor cell apoptosis has often been shown to occur independently of the p53 tumor suppressor gene, which is lost or inactivated in more than half of human malignancies [48]. PARP-1 cleavage is a crucial event that drives death receptor signaling towards apoptosis or necrosis. TNF-induced oxidative stress and DNA damage activate PARP-1, causing ATP depletion and the subsequent production of necrosis [49]. In brief, we demonstrate that Eriodictyol exposure of the cancer cells causes time-dependent upregulation of endogenous TNF-α and TNFRI receptor as well as an increase in expression of the DISC complex components (TNFR1, FADD, and TRADD), leading to activation of cleaved caspase 8, activation of effector caspase 7, thus, ultimately resulting in caspases-dependent apoptotic cell death of the cancer cells. Notably, previous studies have already shown TNFR1-induced, however caspase-independent mode of cell death [50,51]. However, the fact that a pan-caspase inhibitor could not completely block Eriodictyol-induced cell death, hints towards a TNFR1-dependent, yet caspase-independent function of Eriodictyol in the induction of cancer cell death. In addition, Eriodictyol-induced apoptosis is accompanied by cell cycle arrest at the G2/M phase, which is initiated by an increase of γ-H2AX (an indicator of DNA damage) through regulation of Cyclin-B1, Cdc25 and Cdc2. Interestingly, Eriodictyol mediated inhibition of Cdc2 leads to accumulation of cells at the G2M phase, thus, resulting in the accumulation of Cyclin B1. Surprisingly, Eriodictyol causes dramatic inhibition of p21. Some reports suggest p21 promotes carcinogenesis and tumor development by promoting transcription of proteins with mitogenic and anti-apoptotic properties [52–54]. Eriodictyol also inactivates Claspin in cancer cells, which plays a vital role in shifting the tumor cell response from cell cycle arrest to induction of apoptotic cell death in response to prolonged DNA replication arrest or the persistence of DNA damage [55,56]. Finally, assessment of the therapeutic efficacy of Eriodictyol in Balb/c (syngeneic) mouse tumor model, subcutaneously injected with 4T1 mice breast cancer cells, indicates that Eriodictyol treatment results in remarkable regression of tumor volume, tumor weight, and metastatic potential in an in vivo mouse model without any significant adverse effect on animals as confirmed by body weight difference in untreated vs treated mice.

In the present study, we have elucidated the selective anti-cancer mechanism of Eriodictyol through upregulation of TNFR1 along with G2/M arrest and subsequent, activation of apoptosis in cancer cells without affecting the normal cells. Although the mechanism underlying the Eriodictyol-induced upregulation of TNFR1 in cancer cells is still not clearly understood, the preliminary bioinformatics analysis has shown that several probable TFs as critical targets (data not shown). However, investigation of this phenomenon is beyond the scope of the present study and will be focused on in future research. Although natural plant-based compounds including flavonoids have numerous pharmacological benefits, their large-scale extraction from natural sources in high-purity and consistent form, bioavailability, targeted delivery to the site of interest, toxicity and safety are some of the major challenges in medicinal chemistry. Hence, globally, these compounds are under extensive research as important scaffolds and lead for the development of effective and selective chemotherapeutic agents for clinical application. Henceforth, Eriodictyol might necessitate the development of easy to synthesize (biosynthesis using yeast applying promoter adjustment and directed evolution [57]) and non-toxic chemical derivative, designing strategies to improve its bioavailability by increasing absorption and metabolic stability, as well as its targeted delivery by formulating appropriate microencapsulation, nano-delivery system or micro-emulsion [58–63] for improved anti-cancer strategies. To summarize, Eriodictyol has the capability to be a promising lead for the future development of prospective and efficient candidate for successful cancer therapy.

Fig. 10. Depicts the proposed model of Eriodictyol mediated selective cytotoxicity in cancer cells.

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Declaration of Competing Interest statement

The authors declare that there is no conflict of interest.

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Supplementary materials

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Fig. 10. Proposed model of the role of Eriodictyol mediated selective cytotoxicity of cancer cells. The upregulated expression of TNFR1 on cancer cells lead to Eriodictyol mediated apoptosis.
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