

Article

Hesperidin Enhances Doxorubicin Efficacy by Modulating Apoptosis- and Migration-Associated Processes in Human Retinoblastoma Cells

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Simple Summary

Retinoblastoma (RB) is a rare pediatric eye cancer for which chemotherapy remains a cornerstone of treatment, although drug resistance and dose-related toxicity limit therapeutic success. Doxorubicin (DOX) is an effective anticancer agent, but its clinical utility can be improved by compounds that enhance its efficacy at lower doses. Hesperidin (Hes), a naturally occurring flavonoid, has attracted interest due to its biological activity in cancer-related cellular processes. In this study, we investigated whether Hes could enhance the cellular response to DOX in human RB cells under in vitro conditions. Our findings show that the combination treatment increases apoptosis, modulates extracellular matrix (ECM)-associated factors, and suppresses cell migration more effectively than either agent alone. These effects collectively contribute to an enhanced anti-tumor phenotype in RB cells. This work provides a preclinical framework supporting the potential of flavonoid-based chemosensitization strategies and highlights the need for further validation in advanced experimental models.

Abstract

This study evaluated the therapeutic potential and underlying molecular mechanisms of the citrus flavonoid Hes in combination with the chemotherapeutic agent DOX in human RB cells. Cells were treated with Hes alone or in combination with DOX for 24 and 48 h. Hes significantly inhibited cell proliferation and migration and promoted apoptotic cell death, while enhancing the cytotoxic response to DOX under in vitro conditions. Molecular analyses demonstrated that combination treatment markedly modulated ECM-associated markers, including the downregulation of matrix metalloproteinases MMP-2 and MMP-9 and ACTA2 (α -smooth muscle actin, α -SMA), along with the upregulation of tissue inhibitors of metalloproteinases TIMP-1 (tissue inhibitor of metalloproteinases) and TIMP-2. In parallel, the expression of apoptosis-related genes was altered, as evidenced by the upregulation of the B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and Caspase-3 and the downregulation of Bcl-2. Overall, these findings indicate that Hes enhances DOX efficacy by simultaneously engaging apoptotic and migration-associated molecular processes, supporting its potential role as a preclinical chemosensitizing agent that warrants further investigation in advanced experimental models.



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1. Introduction

RB is the most common primary intraocular malignancy of childhood and develops following the inactivation of the Retinoblastoma 1 gene in retinal photoreceptor progenitor cells [1,2]. Current treatment strategies include intravenous chemotherapy, local therapeutic approaches, and intraarterial or intravitreal chemotherapy applications [3,4]. DOX, one of the agents commonly used in systemic chemotherapy, is an anthracycline that exerts potent anticancer activity primarily through inhibition of topoisomerase II. However, its clinical utility is limited by significant adverse effects, including cardiotoxicity, myelosuppression, and the development of drug resistance [5,6]. Consequently, the identification of safer, naturally derived adjuvant compounds capable of enhancing DOX efficacy while potentially reducing dose-related toxicity remains an important clinical objective.

In recent years, naturally derived compounds have attracted increasing interest in cancer chemotherapy and chemoprevention due to their relatively low toxicity profiles and ability to modulate multiple cellular pathways [7,8]. Hes, a flavanone glycoside abundant in citrus fruits, has been widely studied for its antioxidant, anti-inflammatory, anticancer, and neuroprotective properties [9,10]. In various cancer models, including breast, colon, prostate, and hepatocellular carcinoma, Hes has been reported to inhibit cell proliferation, induce apoptosis, suppress angiogenesis, and modulate migration-associated processes [11,12]. These effects have been linked to mechanisms such as cell-cycle arrest, caspase activation, regulation of signaling pathways, and inhibition of ECM related enzymes, including matrix metalloproteinases [13,14].

Despite these promising findings, the effects of Hes in RB, particularly in combination with standard chemotherapeutic agents such as DOX, have not been adequately investigated. Therefore, the present study systematically evaluates the cytotoxic, antiproliferative, and apoptosis-inducing effects of Hes alone and in combination with DOX in the human RB cell line WERI-Rb-1, together with its impact on cell migration and ECM-associated molecular markers. To our knowledge, this is the first comprehensive study examining the combinatorial effects of Hes and DOX in human RB cells. While flavonoid DOX combinations have been explored in several adult solid tumor models, their relevance in RB a rare pediatric intraocular malignancy with distinct biological characteristics, remains largely unexplored.

Furthermore, by analyzing the expression of apoptosis-related genes (Bax, Bcl-2, and Caspase-3) and migration-associated ECM regulators (MMP-2, MMP-9, α -SMA, TIMP-1, and TIMP-2), this study aims to provide mechanistic preclinical insight into the cellular responses elicited by combination treatment. Collectively, these findings are intended to inform the design of future translational and advanced preclinical studies in RB.

2. Materials and Methods

2.1. Reagents and Preparation of Stock Solutions for Cell Culture Experiments

Hes (purity $\geq 97\%$, Sigma-Aldrich, Cat. No. H5254), DOX hydrochloride (Sigma-Aldrich, Cat. No. D1515), and dimethyl sulfoxide (DMSO) used for cell culture experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hes and DOX were prepared as 100 mM stock solutions in DMSO and stored at $-20\text{ }^{\circ}\text{C}$ until use. For all experiments, working solutions were freshly prepared by diluting the stock solutions in culture medium, and the final concentration of DMSO did not exceed 0.1% (v/v).

RPMI-1640 culture medium, fetal bovine serum (FBS), penicillin/streptomycin solution, and trypsin EDTA were obtained from Gibco (Thermo Fisher Scientific, Grand Island, NY, USA). TRIzol reagent and the cDNA synthesis kit were purchased from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA), while SYBR Green qPCR Master Mix was obtained from Applied Biosystems (Thermo Fisher Scientific, Foster City, CA, USA). All gene-specific primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA).

2.2. Cell Culture Conditions and Maintenance of Human RB Cells

The human RB cell line Human retinoblastoma cell line (WERI-Rb-1) (ATCC[®] HTB-169[™], American Type Culture Collection, Manassas, VA, USA) was maintained under continuous culture conditions in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO₂ and 95% relative humidity, as previously described [15]. All experiments were conducted using cells in the logarithmic growth phase when cultures reached approximately 70–80% confluence.

2.3. Experimental Design and Treatment Groups for Single and Combination Treatments

Half maximal inhibitory concentration (IC₅₀)-based concentrations were selected for combination experiments to ensure biologically relevant and comparable levels of cytotoxic stress across different treatment conditions. For cytotoxicity assessment and CI analysis, sub-IC₅₀ concentrations were applied to allow evaluation of interaction effects without complete loss of cell viability. In contrast, IC₅₀-based concentrations were used in migration, apoptosis, and gene expression analyses to ensure robust induction of phenotypic and molecular responses. For combination index (CI) analysis, fixed-ratio dose combinations including concentrations above the single-agent IC₅₀ were intentionally applied, as required by the Chou–Talalay method, and CI values were calculated using dose effect modeling based on mean viability data to enable reliable interaction assessment across low, medium, and high dose levels. Cells were assigned to four experimental groups and treated for 24 or 48 h, with combination treatments primarily applied for 48 h unless otherwise specified.

Control group: Cells treated with complete culture medium containing 0.1% (v/v) DMSO as the vehicle control.

Hes group: Cells treated with Hes at concentrations of 10, 25, 50, and 100 µM.

DOX group: Cells treated with DOX at concentrations of 1, 2.5, 5, and 10 µM.

Combination group: Cells treated with either sub-IC₅₀ concentrations of Hes (25 µM) and DOX (2.5 µM) for interaction analyses or IC₅₀ concentrations of Hes (81.3 µM) and DOX (1.53 µM) in combination for 48 h in mechanistic assays, as specified in the corresponding sections.

2.4. Assessment of Cell Viability Using the MTT Assay

Cell viability was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). In brief, WERI-Rb-1 cells were seeded into 96-well plates at a density of 5×10^3 cells per well and allowed to adhere for 24 h. Cells were then exposed to the indicated treatments for 24 or 48 h. At the end of the treatment period, MTT solution was added to each well at a final concentration of 0.5 mg/mL, and the plates were incubated at 37 °C for 4 h to allow for formazan crystal formation. Subsequently, the culture medium was carefully removed, and DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Multiskan[™] GO, Thermo Fisher Scientific, Vantaa, Finland). Cell viability was calculated as a percentage relative to the mean optical density (OD) of the control group. IC₅₀ values were calculated from dose–response curves generated using nonlinear regression analysis. Cell viability data were plotted as percentage of vehicle control versus logarithmically transformed

drug concentrations. Curve fitting was performed using a four-parameter logistic (4PL) model ($\log[\text{inhibitor}]$ vs. normalized response) implemented in GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). IC_{50} values were defined as the drug concentration required to reduce cell viability by 50% relative to the control condition and were calculated from at least three independent biological experiments.

2.5. CI Analysis

CI analysis was performed to quantitatively evaluate the interaction between Hes and DOX following 48 h of co-treatment. CI values were calculated based on the Chou–Talalay method using dose effect data obtained from the MTT assay. Drug combinations were tested at fixed ratios corresponding to low (Hes 12.5 μM + DOX 1.25 μM), medium (Hes 25 μM + DOX 2.5 μM), and high (Hes 50 μM + DOX 5 μM) dose levels. CI values were interpreted as follows: $\text{CI} < 1$ indicated a greater-than-additive interaction, $\text{CI} = 1$ indicated an additive effect, and $\text{CI} > 1$ indicated an antagonistic interaction. CI calculations were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) based on mean viability values derived from at least three independent biological experiments.

2.6. Assessment of Cell Migration Using the Wound Healing Assay

Cell migration ability was analyzed using a scratch (wound healing) assay. For combination and mechanistic analyses, a 48 h treatment period was selected based on the observed time-dependent cytotoxic responses and to allow sufficient manifestation of transcriptional and phenotypic changes. For migration assessment, WERI-Rb-1 cells were seeded at a density of 5×10^5 cells per well into 6-well plates and grown to 100% confluence. A straight scratch was created across the cell monolayer using a sterile 200 μL pipette tip. Cellular debris was removed by washing with phosphate-buffered saline, and treatment agents prepared in serum free medium were applied. The scratch area was photographed immediately after scratching (0 h) and after 48 h using a phase contrast inverted microscope (model unspecified, Olympus Corporation, Tokyo, Japan). Cell migration was quantified by calculating the percentage of wound closure using ImageJ software (version 1.53, National Institutes of Health, Bethesda, MD, USA).

2.7. Flow Cytometric Analysis of Apoptosis Using Annexin V/Propidium Iodide (PI) Staining

Apoptosis induction was quantified using flow cytometry following staining with fluorescein isothiocyanate-conjugated Annexin V and PI. After the indicated treatments, cells were harvested, washed with Annexin V binding buffer, and incubated with FITC-Annexin V and PI for 15 min at room temperature in the dark, according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

Stained cells were analyzed using a flow cytometer (FACSCanto™ II, BD Biosciences, San Jose, CA, USA). Initial gating was performed to exclude cellular debris and doublets based on forward scatter (FSC) and side scatter (SSC) characteristics. Cell populations were classified as viable (Annexin V[−]/PI[−]), early apoptotic (Annexin V⁺/PI[−]), late apoptotic (Annexin V⁺/PI⁺), and necrotic (Annexin V[−]/PI⁺) based on fluorescence intensity.

2.8. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Genes were selected based on their established involvement in apoptosis execution, ECM remodeling, and migration-associated cellular processes, in accordance with the functional assays performed in this study. Total cellular RNA was isolated using TRIzol™ reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For gene expression

analyses, WERI-Rb-1 cells were treated with Hes (81.3 μ M) and DOX (1.53 μ M), corresponding to their respective 48 h IC₅₀ values, either alone or in combination for 48 h prior to RNA isolation.

Reverse transcription was performed using 1 μ g of total RNA with a cDNA synthesis kit and random hexamer primers (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Gene-specific primer sequences for BAX, BCL2, CASP3, MMP2, MMP9, α -SMA (ACTA2), TIMP1, TIMP2, and the reference gene ACTB are listed in Table 1. Quantitative real-time PCR was carried out using SYBRTM Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) on a StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Table 1. Primer sequences used in qRT-PCR.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
MMP2	TCTCCTGACATTGACCTTGGC	CAAGGTGCTGGCTGAGTAGATC
MMP9	GCCACTACTGTGCCTTTGAGTC	CCCTCAGAGAATCGCCAGTACT
ACTA2	CTATGCCTCTGGACGCACAACT	CAGATCCAGACGCATGATGGCA
TIMP1	GGAGAGTGTCTGCGGATACTTC	GCAGGTAGTGATGTGCAAGAGTC
TIMP2	TGCAACTCGGACCTGGTTAT	CCTTTGATGCAGGCGTAGAG
BAX	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC
BCL2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
CASP3	GGAAGCGAATCAATGGACTCTGG	GCATCGACATCTGTACCAGACC
ACTB	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG

The amplification protocol consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at the primer-specific temperature for 60 s. The specificity and quality of PCR products were confirmed by melt curve analysis, which demonstrated a single, specific amplification peak for each primer pair. PCR amplification efficiency was verified during primer optimization and was consistent across experiments. ACTB was selected as the reference gene based on its minimal Ct variability across all experimental groups and treatment conditions, supporting its suitability as an internal control in the present experimental system. Although α -SMA and ACTB encode cytoskeletal proteins, α -SMA expression was assessed relative to ACTB to enable consistent normalization across all target genes, and the results were interpreted in conjunction with functional migration and phenotypic assays rather than as an isolated transcriptional readout. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to the control group [16].

2.9. Bioinformatic Analyses

Bioinformatic analyses were performed in an exploratory and supportive manner to contextualize the qRT-PCR results within known biological pathways. These analyses were not intended as transcriptome-wide (RNA-seq or omics-level) investigations and were not designed for novel gene discovery or statistical inference. Instead, they were restricted to the eight genes experimentally quantified by qRT-PCR and aimed solely to associate the observed expression changes with relevant biological pathways reported in the literature. Accordingly, the analyses should be interpreted as targeted pathway annotation or contextual pathway analysis rather than classical pathway enrichment.

The input gene set consisted exclusively of genes whose expression was directly measured in this study, including Bax, Bcl-2, Caspase-3, MMP-2, MMP-9, ACTA2 (α -SMA), TIMP-1, and TIMP-2. These genes were selected based on their established roles in apoptosis execution, extracellular matrix remodeling, cell adhesion, and migration-associated cellular processes, in alignment with the functional assays performed. To provide biological context,

functional information related to the experimentally measured genes was retrieved from publicly available databases and the literature to support pathway-level interpretation, without expanding the experimentally measured gene set or implying protein–protein interaction or network analysis.

Gene Ontology (GO) and KEGG pathway annotations were conducted using STRING (version 12.0; <https://string-db.org>), DAVID (version 6.8; <https://davidbioinformatics.nih.gov>), and SRPLOT (<https://www.bioinformatics.com.cn/en>), all accessed on 10 December 2025. Only descriptive pathway associations were evaluated, without the use of enrichment scores, gene counts, statistical ranking metrics, or protein–protein interaction analyses.

2.10. Statistical Analysis

All experiments were performed in at least three independent biological experiments. Data are presented as mean $\pm$ standard deviation (SD). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism version 9.0 software (GraphPad Software, San Diego, CA, USA). A p -value < 0.05 was considered statistically significant. Prior to ANOVA, data distribution normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test.

3. Results

3.1. Single-Agent Cytotoxic Effects of Hes and DOX in WERI-Rb-1 Cells

The cytotoxic effects of hesperidin (Hes) and doxorubicin (DOX) on WERI-Rb-1 cell viability were evaluated using the MTT assay following 24 and 48 h of treatment. Both agents induced a statistically significant reduction in cell viability in a dose and time-dependent manner ($p < 0.05$); however, their cytotoxic potency and temporal profiles differed markedly.

Hes treatment did not reduce cell viability below 50% at concentrations up to 100 μ M after 24 h, and therefore the 24 h IC_{50} value was estimated to be >100 μ M (Figure 1A). In contrast, prolonged exposure for 48 h resulted in a measurable IC_{50} value of approximately 81.3 μ M (Figure 1B), indicating a time-dependent increase in cytotoxicity. At low to moderate concentrations (10–25 μ M), Hes exerted limited cytotoxic effects, whereas a pronounced reduction in viability was observed at higher concentrations.

DOX exhibited substantially greater cytotoxic potency compared to Hes at both time points. The IC_{50} value for DOX was calculated to be approximately 3.64 μ M after 24 h and decreased to 1.53 μ M following 48 h of treatment (Figure 1D,E). Notably, during the 48 h incubation period, DOX concentrations of 2.5 μ M and above resulted in a sharp decline in cell viability, highlighting the strong time-dependent cytotoxic activity of this agent.

For clearer visualization of dose–response relationships and IC_{50} determination, the corresponding curves plotted on a logarithmic concentration scale are provided in Supplementary Figure S1. The IC_{50} values for Hes and DOX at 24 h and 48 h are summarized in Table 2. Collectively, these findings demonstrate that Hes displays comparatively lower and delayed cytotoxic effects, whereas DOX induces a rapid and pronounced reduction in WERI-Rb-1 cell viability. This distinct difference in cytotoxic profiles provided the basis for subsequent combination treatment and interaction analyses.

Table 2. IC_{50} values of Hes and DOX in WERI-Rb-1 cells following 24 h and 48 h treatment.

Compound	Treatment Time	IC_{50} (μ M)	Notes
Hes	24 h	>100 μ M	Cell viability did not decrease below 50% within the tested concentration range
Hes	48 h	81.3 μ M	Calculated from dose–response curve

Table 2. Cont.

Compound	Treatment Time	IC ₅₀ (μM)	Notes
DOX	24 h	3.64 μM	Calculated from dose–response curve
DOX	48 h	1.53 μM	Calculated from dose–response curve

Data represent mean values derived from $n = 3$ independent biological experiments. IC₅₀ values were determined using nonlinear regression analysis of dose–response curves generated from MTT assay results.

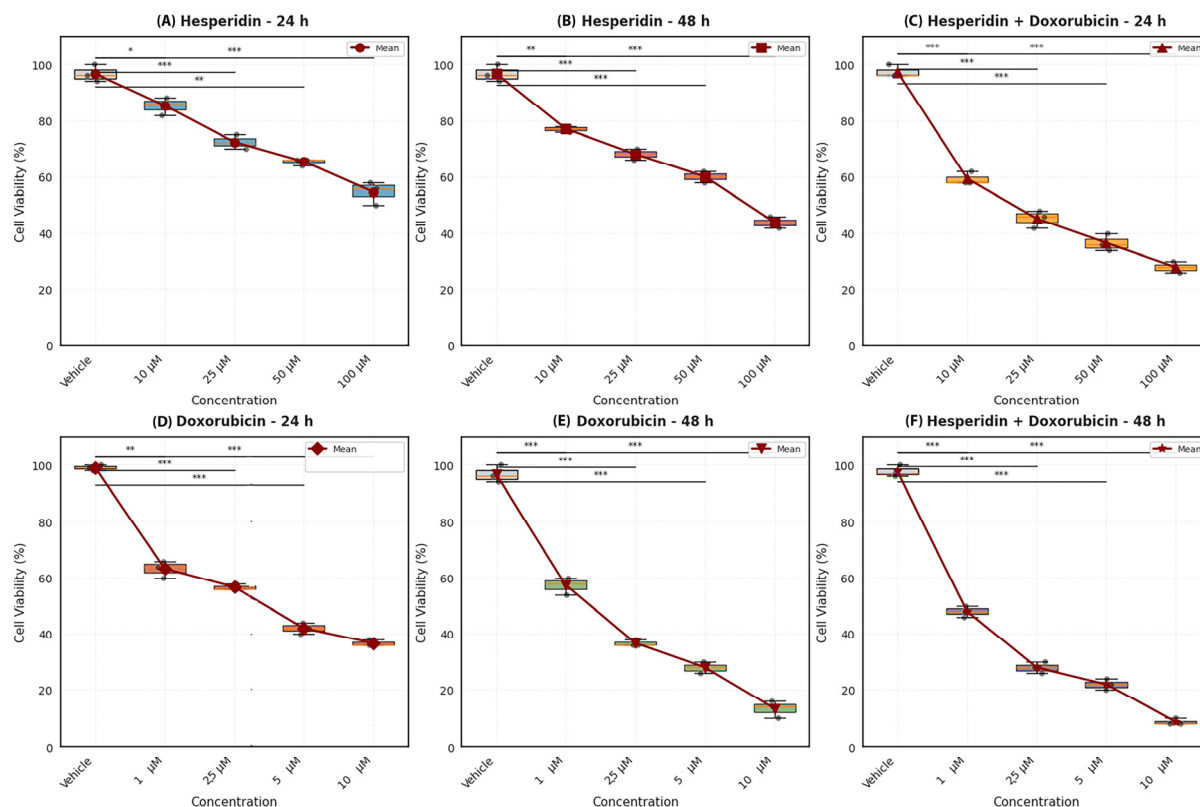


Figure 1. Effects of Hes, DOX, and their combination on the viability of WERI-Rb-1 cells. WERI-Rb-1 cells were treated with increasing concentrations of Hes (A,B), DOX (D,E), or their fixed-ratio combination (C,F) for 24 h (A,C,D) and 48 h (B,E,F). Cell viability is expressed as a percentage of the vehicle control. Box plots represent the distribution of $n = 3$ independent biological experiments, with the central line indicating the median and whiskers indicating data dispersion; overlaid symbols and connecting lines indicate group mean values. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle control.

3.2. Combination Treatment Enhances Cytotoxicity and Induces a Greater-than-Additive Interaction

To determine whether Hes enhances the cytotoxic response to DOX, cell viability following combination treatment was analyzed and compared with single-agent exposure. Combination treatment data are presented in Figure 1C,F. At 48 h, DOX administered alone exhibited an IC₅₀ value of approximately 1.53 μM, whereas co-treatment with Hes (25 μM) achieved an equivalent cytotoxic effect at a lower effective DOX concentration range, indicating a leftward shift in the DOX dose–response curve under combination conditions.

Specifically, during the 48 h incubation period, the Hes (25 μM) + DOX (2.5 μM) combination resulted in a significantly greater reduction in cell viability compared to DOX alone (Figure 1F). The extent of cytotoxicity observed in the combination group exceeded that produced by either agent administered individually, consistent with a greater-than-additive interaction under the defined experimental conditions.

To quantitatively assess this interaction, CI analysis was performed following 48 h of co-treatment (Figure 2). CI values below 1 were consistently observed across the tested dose combinations, confirming a greater-than-additive effect of Hes and DOX on WERI-Rb-1 cell viability. Based on these results, a sub-IC₅₀ combination of Hes (25 µM) and DOX (2.5 µM) was selected for interaction analyses, while IC₅₀-based concentrations were applied in subsequent migration, apoptosis, and gene expression experiments.

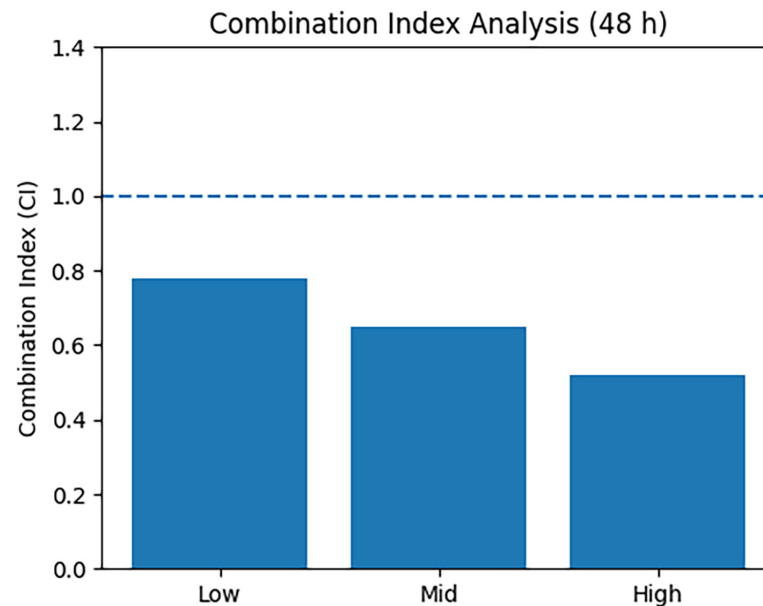


Figure 2. CI analysis of Hes and DOX co-treatment in WERI-Rb-1 cells after 48 h. CI values were calculated according to the Chou–Talalay method for fixed-ratio combinations at low (Hes 12.5 µM + DOX 1.25 µM), medium (Hes 25 µM + DOX 2.5 µM), and high (Hes 50 µM + DOX 5 µM) dose levels. CI values represent mean indices derived from $n = 3$ independent biological experiments. The dashed horizontal line indicates CI = 1 (additive effect), whereas CI values below 1 indicate a greater-than-additive interaction under the defined experimental conditions.

Together, these findings demonstrate that Hes enhances the cytotoxic efficacy of DOX in WERI-Rb-1 cells and support the use of this combination strategy for further mechanistic investigations.

3.3. Combination Treatment Markedly Suppresses Two-Dimensional Cell Migration in WERI-Rb-1 Cells

Wound healing assays were performed to evaluate the effects of Hes and DOX treatments on two-dimensional collective cell migration under in vitro conditions. After 48 h of treatment, both IC₅₀ Hes and IC₅₀ DOX significantly reduced the wound closure rate compared to the control group ($p < 0.01$). The most pronounced inhibition was observed in the combination group, in which cell migration was almost completely suppressed. In this group, an approximate 85% reduction in wound closure was recorded relative to the control group ($p < 0.001$) (Figure 3).

Collectively, these results demonstrate a marked anti-migratory effect and suppression of migration-associated phenotypic features under in vitro conditions, rather than direct evidence of metastatic inhibition.

Consistent with the quantitative migration data, representative phase-contrast micrographs from the wound healing assay further illustrate the treatment-dependent differences in collective cell migration (Figure 4). At 48 h, control cells exhibited pronounced wound closure, whereas Hes- and DOX-treated cells showed a partial reduction in migratory capacity. In contrast, the Hes + DOX combination group displayed a markedly

wider residual wound area, indicating near-complete suppression of wound closure under in vitro conditions. These representative images visually corroborate the quantified wound closure and migration inhibition values presented above, confirming that combination treatment exerts a strong anti-migratory effect rather than promoting wound repair-associated cell movement.

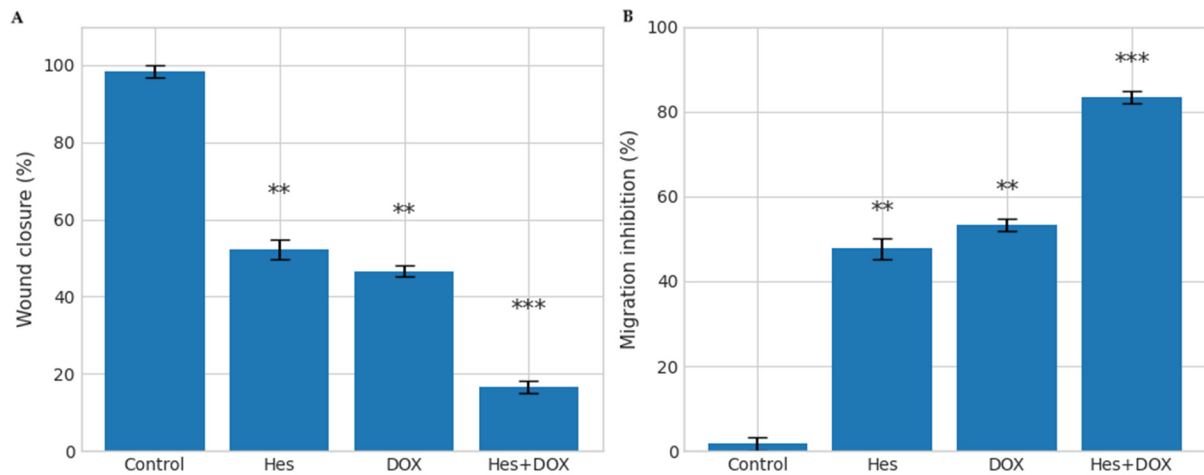


Figure 3. Effects of Hes, DOX, and their combination on WERI-Rb-1 cell migration assessed by the wound healing assay after 48 h. **(A)** Quantification of wound closure expressed as percentage of the initial wound area. **(B)** Migration inhibition calculated relative to the control group. Data are presented as mean $\pm$ SD from $n = 3$ independent biological experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test (** $p < 0.01$, *** $p < 0.001$ versus control).

3.4. Combination Treatment Modulates Migration-Associated ECM Gene Expression

mRNA expression levels of genes associated with cell migration and ECM remodeling were analyzed by qRT-PCR. Treatment with DOX alone resulted in a modest increase in MMP-2 and MMP-9 expression, whereas co-treatment with Hes significantly suppressed this increase ($p < 0.01$). The most pronounced reduction was observed in the combination treatment group, in which MMP-2 and MMP-9 expression levels were approximately 4-fold and 5-fold lower, respectively, compared to the control group ($p < 0.001$ for both) (Figure 5A,B).

In parallel, treatment-dependent changes were also observed in the expression of tissue inhibitors of metalloproteinases. While DOX alone slightly increased TIMP-1 expression, combination treatment resulted in a significant upregulation of both TIMP-1 and TIMP-2 ($p < 0.05$ and $p < 0.01$, respectively) (Figure 5C,D).

Importantly, transcriptional analysis revealed significant downregulation of ACTA2 (α -SMA) expression following combination treatment. While DOX or Hes alone caused only moderate changes, co-treatment with Hes and DOX led to a marked reduction in ACTA2 mRNA levels compared to the control group ($p < 0.01$), indicating suppression of a migratory and contractile cellular phenotype (Figure 5E).

3.5. Combination Treatment Potentiates Apoptosis-Related Gene Expression in WERI-Rb-1 Cells

The effects of Hes, DOX, and combination treatments on apoptosis-related molecular mechanisms were evaluated in WERI-Rb-1 cells using qRT-PCR analysis (Figure 6A–C). Compared to the control group, DOX treatment alone resulted in a significant increase in Bax mRNA expression ($p < 0.01$). Hes treatment induced a moderate but statistically significant increase in Bax expression compared to the control group ($p < 0.05$). The most pronounced increase in Bax expression was observed in the Hes + DOX combination group,

where expression levels were approximately 3–4 fold higher than those in the control group ($p < 0.001$) (Figure 6A).

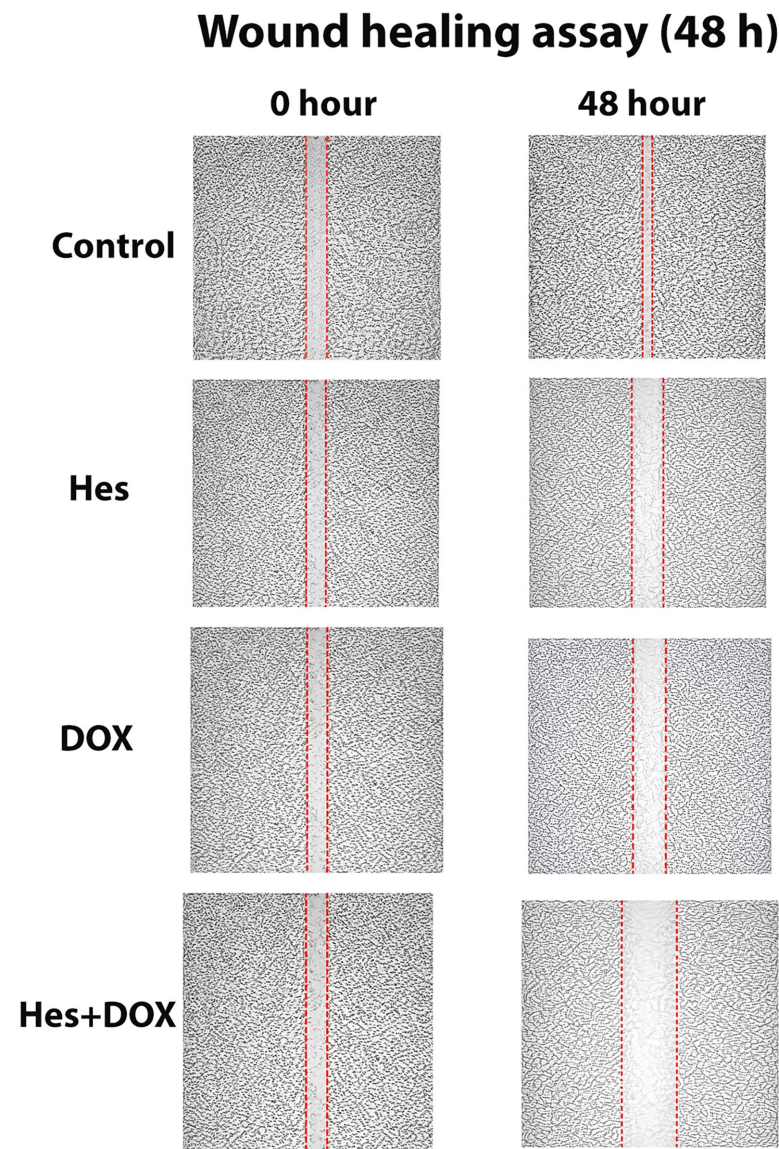


Figure 4. Representative phase-contrast images of the wound healing assay showing the two-dimensional collective migration of WERI-Rb-1 cells following treatment with Hes, DOX, and their combination. Images were acquired at 0 h and 48 h after scratch generation. Red dashed lines indicate wound boundaries used for quantitative analysis. Control cells exhibit substantial wound closure at 48 h, whereas Hes and DOX-treated cells show partial closure. The Hes + DOX combination preserves a wide wound gap, consistent with strong inhibition of cell migration.

In parallel, anti-apoptotic Bcl-2 mRNA expression was significantly reduced following DOX treatment compared to the control group ($p < 0.01$). While Hes alone caused a moderate suppression of Bcl-2 expression ($p < 0.05$), combination treatment reduced Bcl-2 levels to their lowest values, corresponding to an approximate 60–70% decrease relative to the control group ($p < 0.001$) (Figure 6B).

Caspase-3 mRNA expression, an effector marker of apoptosis, was significantly increased following DOX treatment compared to the control group ($p < 0.01$). Hes treatment resulted in a moderate increase in Caspase-3 expression ($p < 0.05$), whereas the combination group exhibited the highest expression levels, with an approximately fourfold increase compared to the control group ($p < 0.001$) (Figure 6C).

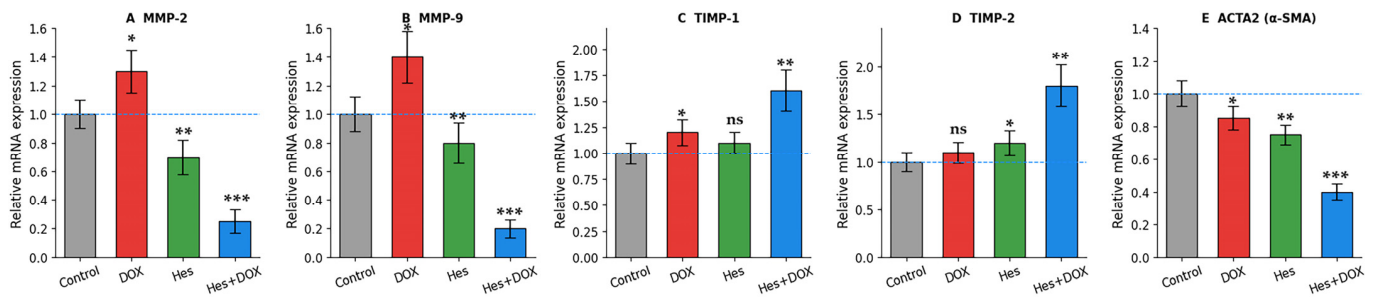


Figure 5. Effects of Hes, DOX, and their combination on the expression of migration- and ECM-associated genes in WERI-Rb-1 cells. Relative mRNA expression levels of MMP-2 (A), MMP-9 (B), TIMP-1 (C), TIMP-2 (D), and ACTA2 (E) were quantified by qRT-PCR and normalized to the control group. Data are presented as mean $\pm$ SD from $n = 3$ independent biological experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control. The blue dashed line indicates the normalized control level (relative expression = 1.0) (ns: not significant).

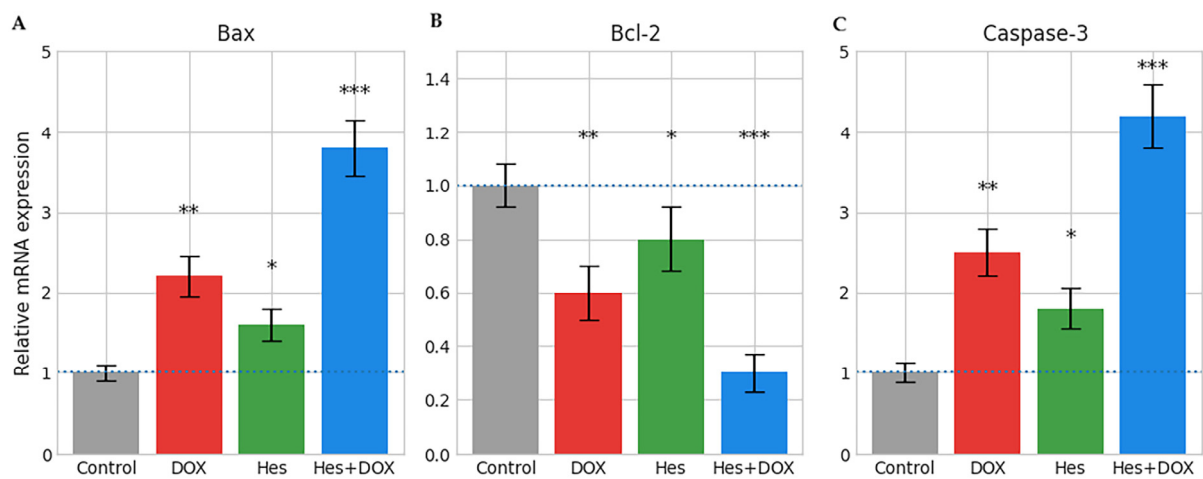


Figure 6. Effects of Hes, DOX, and their combination on the expression of apoptosis-associated genes in WERI-Rb-1 cells. Relative mRNA expression levels of Bax (A), Bcl-2 (B), and Caspase-3 (C) were quantified by qRT-PCR and normalized to the control group. Data are presented as mean $\pm$ SD from $n = 3$ independent biological experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control. The blue dashed line indicates the normalized control level (relative expression = 1.0).

Collectively, combination treatment shifted the Bax/Bcl-2 expression balance toward a pro-apoptotic profile by concurrently enhancing Bax and Caspase-3 expression and suppressing Bcl-2 levels. These coordinated molecular changes indicate activation of apoptosis-related pathways under in vitro conditions. Consistent with these transcriptional findings, Annexin V/PI flow cytometric analysis revealed a clear treatment-dependent redistribution of cell populations, with an increased proportion of early and late apoptotic cells following Hes and DOX exposure and a distinct apoptotic profile under combination treatment conditions (Figure 7). Together, these results provide complementary molecular and cellular-level evidence supporting apoptosis induction under combination treatment conditions, as described in Section 2.7.

3.6. Targeted Bioinformatic Analyses to Contextualize Experimentally Observed Molecular Changes

To provide biological context for the experimentally measured gene expression changes, a targeted pathway annotation analysis was conducted using the eight genes quantified by qRT-PCR. As this analysis was descriptive in nature, no numerical enrichment

parameters (such as gene counts, enrichment scores, or rich factors) were applied. The associated KEGG pathways primarily included apoptosis-related signaling, oxidative stress response, cell cycle regulation, and cytoskeletal organization, which are conceptually consistent with the observed effects of hesperidin and doxorubicin treatments. These findings do not imply the involvement of additional genes beyond those experimentally measured but rather support the biological relevance of the selected gene panel.

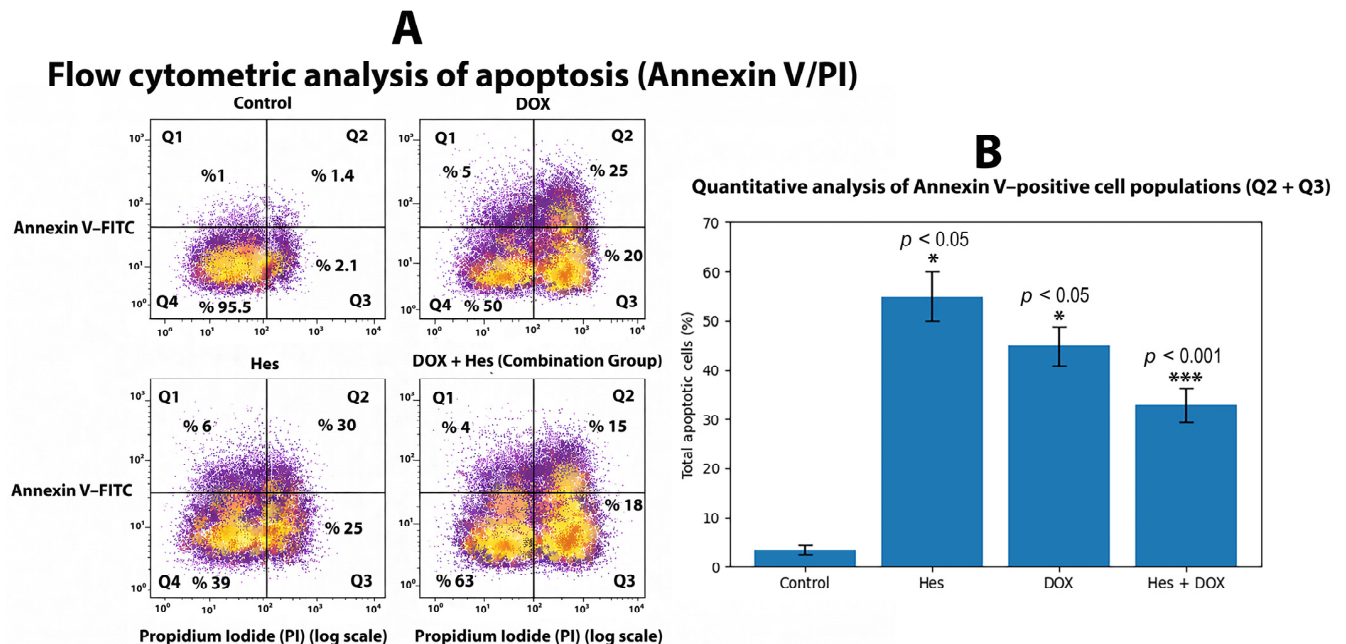


Figure 7. Flow cytometric analysis of Annexin V/PI staining in WERI-Rb-1 cells following Hes, DOX, and combination treatment. **(A)** Representative Annexin V-FITC/propidium iodide (PI) dot plots illustrating the distribution of cell populations in control, Hes, DOX, and Hes + DOX-treated cells after 48 h. Quadrants indicate Q1 (PI⁺/Annexin V[−]; necrotic), Q2 (Annexin V⁺/PI⁺), Q3 (Annexin V⁺/PI[−]; early apoptotic), and Q4 (Annexin V[−]/PI[−]; viable) cell populations. Quadrant percentages sum to 100% for each experimental condition. Lighter/yellow colors indicate higher cell density, whereas darker/purple colors indicate lower cell density. **(B)** Quantitative analysis of Annexin V-positive cell populations, calculated as the sum of Q2 (Annexin V⁺/PI⁺) and Q3 (Annexin V⁺/PI[−]) populations. Data are presented as mean $\pm$ SD from $n = 3$ independent biological experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test (* $p < 0.05$, *** $p < 0.001$ versus control).

GO biological process annotation was applied to this experimentally defined and functionally curated gene set to identify biological themes consistent with the observed in vitro phenotypes. The identified biological processes were primarily related to the regulation of programmed cell death, negative regulation of cell migration, extracellular matrix organization, and cell adhesion (Figure 8). These functional categories aligned closely with the transcriptional modulation of apoptosis- and migration-associated markers as well as with the phenotypic findings obtained from apoptosis and wound healing assays.

The identified pathways reflect biological processes that are conceptually consistent with the observed experimental outcomes. Pathway visualization was used solely to present KEGG pathway categories associated with the experimentally measured gene set in a descriptive manner (Figure 9).

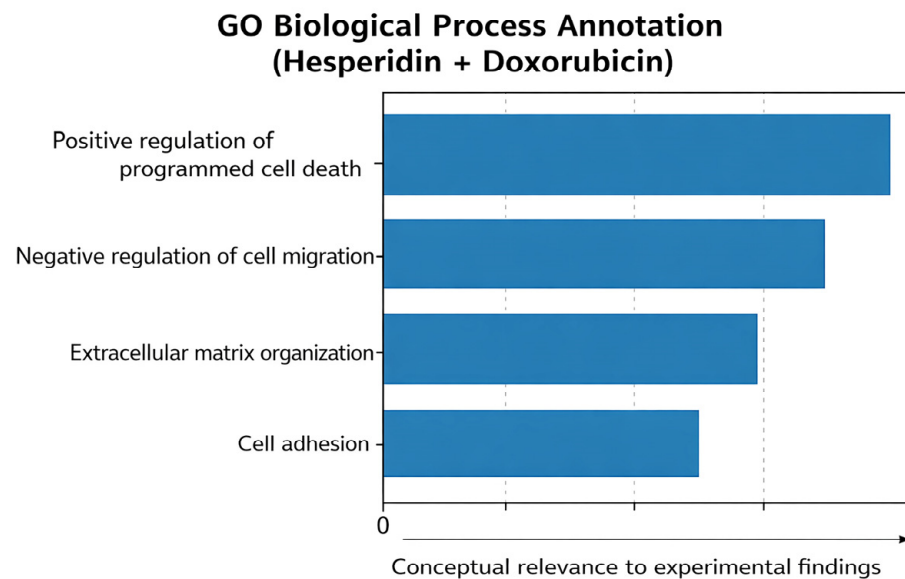


Figure 8. GO biological process annotation of an experimentally defined, targeted gene set associated with Hes and DOX combination treatment in WERI-Rb-1 cells. The identified biological processes are presented descriptively to provide functional context for experimentally observed apoptosis- and migration-associated molecular changes, without implying statistical enrichment or transcriptome-wide analysis.

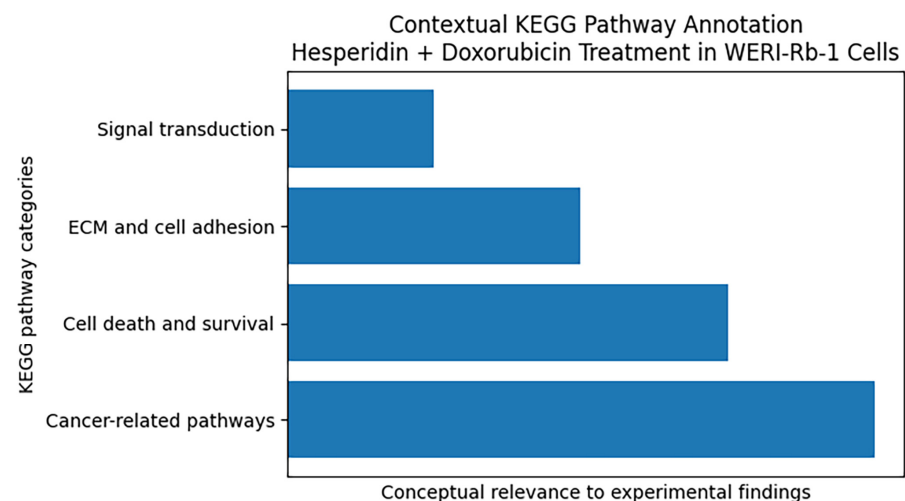


Figure 9. Contextual KEGG pathway annotation of the experimentally measured qRT-PCR gene set in Hesperidin + Doxorubicin-treated WERI-Rb-1 cells. The pathways are presented descriptively to illustrate their conceptual alignment with the observed experimental findings.

KEGG pathway annotation was applied to the same targeted gene set to provide pathway-level context and biological interpretation. The identified pathways were primarily related to apoptosis-associated signaling, extracellular matrix receptor interaction, cell adhesion, and cancer-related processes, all of which were concordant with the experimental findings obtained from cytotoxicity, migration, apoptosis, and gene expression analyses (Figure 10). Given the limited and targeted nature of the analyzed gene set, these pathway associations were interpreted descriptively and used solely to support and contextualize the experimentally observed molecular and phenotypic changes rather than to generate independent predictive conclusions.

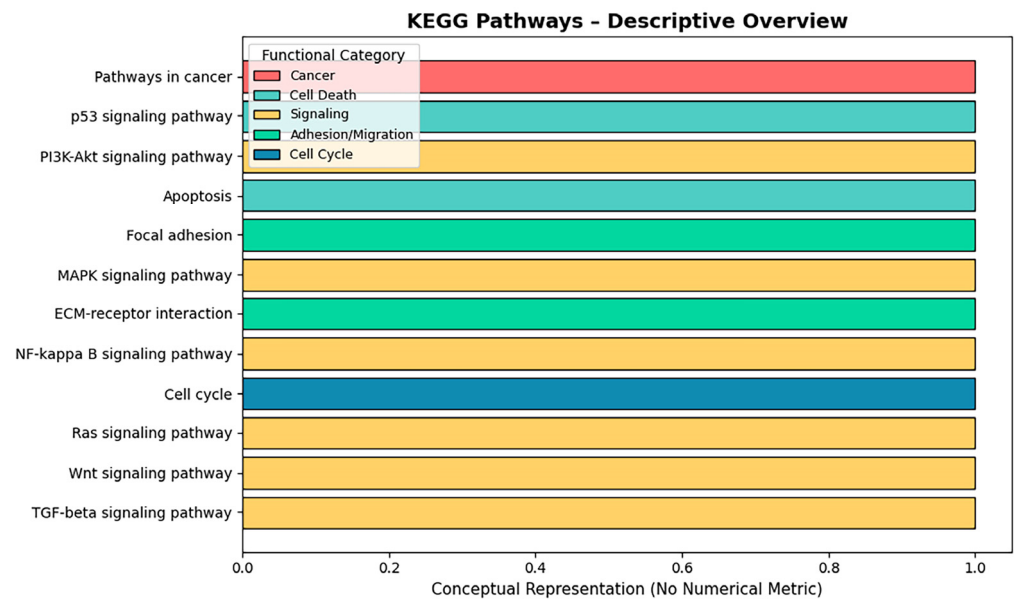


Figure 10. Contextual KEGG pathway annotation based on the experimentally measured qRT-PCR gene set. The pathways are presented descriptively to illustrate their conceptual alignment with the observed experimental findings.

4. Discussion

In the present study, we investigated the effects of the citrus flavonoid Hes in combination with the anthracycline chemotherapeutic agent DOX in human retinoblastoma (WERI-Rb-1) cells under in vitro conditions. Our findings demonstrate that Hes enhances the cytotoxic, anti-migratory, and pro-apoptotic responses to DOX through coordinated modulation of molecular processes associated with cell survival, ECM organization, and cell migration. Unlike studies that primarily focus on isolated endpoints such as cytotoxicity or apoptosis alone, the present work integrates cell viability, migration-related phenotypic outcomes, and transcriptional regulators of ECM remodeling, including MMP-2, MMP-9, TIMP-1, TIMP-2, and α -SMA, thereby providing a more comprehensive framework for interpreting the cellular responses elicited by Hes + DOX combination treatment.

Analysis of cell viability revealed distinct pharmacological profiles for each agent. Hes alone exhibited moderate cytotoxicity, with a 48 h IC_{50} value of 81.3 μ M, consistent with its reported low toxicity in both normal and malignant cell models [17,18]. In contrast, DOX demonstrated potent single-agent activity, with IC_{50} values of 3.64 μ M at 24 h and 1.53 μ M at 48 h, in line with its established efficacy in retinoblastoma treatment [4]. Notably, the leftward shift in the DOX dose–response curve observed under combination conditions indicates a greater-than-additive cytotoxic effect. This enhanced response may reflect flavonoid-mediated modulation of intracellular drug handling and cellular stress responses, as previously suggested for flavonoid chemotherapeutic combinations [19–21].

The enhanced cellular response induced by combination treatment was further reflected in migration assays. While Hes and DOX individually reduced wound closure, their combination resulted in near-complete suppression of two-dimensional collective cell migration under in vitro conditions. This phenotypic inhibition was accompanied by coordinated transcriptional downregulation of MMP-2 and MMP-9 together with upregulation of their endogenous inhibitors TIMP-1 and TIMP-2, suggesting reduced ECM-degrading capacity. Suppression of ACTA2 expression is consistent with the observed inhibition of cell migration [22,23].

At the apoptotic level, combination treatment promoted a pronounced shift toward pro-apoptotic signaling. Increased Bax expression, concomitant suppression of Bcl-2, and

upregulation of Caspase-3 collectively indicate engagement of intrinsic apoptosis-related pathways [24,25]. Although molecular analyses were performed at the mRNA expression level without direct protein level validation, the selected apoptosis and migration-associated targets represent well characterized regulators whose transcriptional modulation has been widely reported to correlate with functional cellular outcomes in cancer models [22,26–28]. Importantly, in the present study, these transcriptional changes were consistently supported by phenotypic assays, including reduced cell viability, enhanced apoptotic cell death, and marked suppression of cell migration.

Consistent with the present findings, a growing body of evidence indicates that flavonoids can act as effective chemosensitizing agents when combined with anthracycline-based chemotherapeutics. A comprehensive review by Asnaashari et al. demonstrated that flavonoids modulate doxorubicin efficacy across multiple cancer models by influencing apoptosis-related signaling cascades, caspase activation, and cell cycle regulation, thereby enhancing chemotherapy-induced cell death while potentially mitigating treatment-associated toxicity [19]. Experimental studies have further validated the chemosensitizing potential of hesperidin in metastatic breast cancer models, where it synergistically enhanced doxorubicin-induced cytotoxicity, promoted apoptotic cell death, induced G2/M cell cycle arrest, and counteracted doxorubicin-induced migratory behavior through downregulation of Rac-1 and MMP-9 expression [29].

Beyond combination-specific studies, accumulating experimental evidence supports a broader role for flavonoids in suppressing tumor cell migration and invasion through the coordinated regulation of matrix metalloproteinases. In this context, Du et al. demonstrated that specific flavonoid compounds significantly inhibited cancer cell migration and invasion via downregulation of MMP-2 and MMP-9 at both the transcriptional and protein levels [30]. Complementary reviews have highlighted flavonoids as key modulators of MMP expression and activity, thereby reinforcing ECM integrity and limiting metastatic progression across diverse solid tumor types [31].

Although data specifically addressing flavonoid doxorubicin combinations in retinoblastoma models remain limited, current molecular insights into retinoblastoma biology suggest that conserved cellular stress response, apoptotic, and survival pathways are central drivers of tumor behavior. Comprehensive analyses of the molecular landscape of retinoblastoma underscore the involvement of core regulatory networks governing cell cycle control, apoptosis, and therapeutic responsiveness, supporting the biological plausibility that combination strategies targeting these conserved mechanisms may elicit comparable cellular responses across tumor contexts [32].

From a translational perspective, the use of Hes as a chemosensitizing agent may offer practical advantages. As a dietary flavonoid with an established safety profile, Hes may enhance the cellular response to DOX while potentially allowing dose optimization to mitigate treatment-associated toxicity. Although the present findings are limited to in vitro conditions, they provide a preclinical framework supporting further investigation of Hes + DOX combination strategies in advanced experimental models.

In summary, the present study provides an integrated preclinical evaluation of Hes as a chemosensitizing agent that enhances DOX efficacy in human retinoblastoma cells through the coordinated modulation of apoptosis-, ECM-, and migration-associated processes. The combination treatment consistently promoted a pro-apoptotic shift while simultaneously suppressing ECM remodeling and cellular migration. A conceptual schematic model (Figure 11) summarizes this integrative framework and is presented as a hypothesis-generating representation rather than evidence of direct causal signaling relationships. Collectively, these results establish a coherent experimental and conceptual basis for further investigation of flavonoid-based chemosensitization strategies in retinoblastoma.

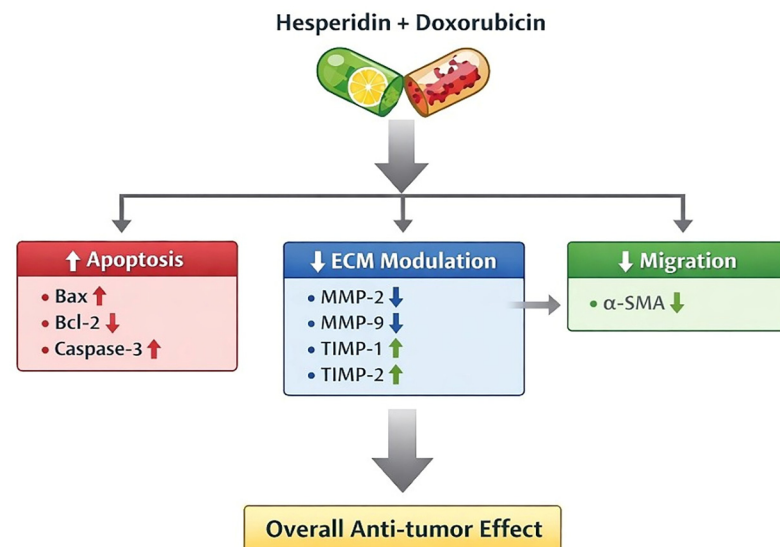


Figure 11. Conceptual overview of the cellular responses induced by combined Hes and DOX treatment in WERI-Rb-1 cells. The schematic illustrates the main experimentally observed cellular outcomes following combination treatment, including activation of apoptosis-related markers, modulation of ECM-associated factors, and suppression of migratory behavior. The diagram summarizes the directional changes in key molecular readouts measured in this study and their integration into an overall anti-tumor response. Arrows represent associative relationships based on *in vitro* findings and are intended for visual synthesis of the results rather than depiction of direct mechanistic causality.

Despite the robust *in vitro* findings presented in this study, several important limitations should be acknowledged. First, hesperidin is known to exhibit limited oral bioavailability and suboptimal pharmacokinetic properties, which may restrict its direct translational applicability. Accordingly, the concentrations employed under *in vitro* conditions may not be readily achievable *in vivo* without formulation optimization or advanced delivery strategies. Second, the present work represents a strictly *in vitro* preclinical proof-of-concept conducted in a single human retinoblastoma cell line. While this model provides valuable mechanistic insight, it does not recapitulate the complexity of the tumor microenvironment, pharmacokinetics, pharmacodynamics, or systemic toxicity profiles observed *in vivo*. In addition, the selectivity of the Hes + DOX combination toward malignant versus non-malignant retinal cells was not evaluated and warrants further investigation. The absence of protein level validation for the analyzed molecular markers constitutes an additional limitation and should be addressed in future studies using complementary approaches such as immunoblotting or immunofluorescence-based analyses. In the present study, relative gene expression analyses were normalized to a single reference gene. While stable expression was observed across the experimental conditions analyzed, the use of multiple validated reference genes may further improve normalization accuracy and analytical robustness in future investigations. Furthermore, the lack of validation across additional retinoblastoma cell lines and the absence of *in vivo* animal model experiments limit the generalizability of the present findings and should be addressed in subsequent studies. Finally, although the study identifies coordinated molecular changes associated with apoptosis induction, migration suppression, and ECM modulation, direct causal mechanisms such as intracellular drug handling, oxidative stress dynamics, or long-term adaptive responses were not dissected. Moreover, wound healing assays model two-dimensional collective cell migration and do not fully recapitulate the complexity of *in vivo* tumor dissemination. Therefore, the observed effects should be interpreted as anti-migratory and ECM-modulatory rather than as direct anti-metastatic outcomes. Future studies employing

three-dimensional culture systems, patient-derived models, and in vivo retinoblastoma models will be essential to validate the biological relevance, therapeutic window, and translational feasibility of the Hes + DOX combination strategy.

5. Conclusions

In conclusion, this study provides a preclinical evaluation of Hes as a chemosensitizing agent that enhances the cellular response to DOX in human retinoblastoma cells under in vitro conditions. By integrating phenotypic assays and molecular analyses, the findings demonstrate that the Hes + DOX combination consistently amplifies cytotoxicity while concurrently promoting apoptosis and suppressing migration-associated ECM remodeling. The combination treatment induced a coordinated pro-apoptotic shift, reflected by increased Bax and Caspase-3 expression together with suppression of Bcl-2, while simultaneously stabilizing ECM dynamics through downregulation of MMP-2, MMP-9, and α -SMA and upregulation of TIMP-1 and TIMP-2. Rather than relying on a single dominant mechanism, the observed effects support a model in which Hes potentiates DOX efficacy through parallel and convergent modulation of apoptosis-related signaling and ECM-regulated migratory behavior. Supportive bioinformatic analyses were used to contextualize these experimentally observed molecular and phenotypic changes within established functional frameworks, without implying direct causal or hierarchical regulatory relationships. Although limited to in vitro conditions, the present study establishes a coherent experimental foundation supporting flavonoid-based chemosensitization as a rational strategy in retinoblastoma research. Future studies employing advanced three-dimensional culture systems and in vivo models will be required to further evaluate the biological relevance, safety profile, and translational feasibility of the Hes + DOX combination approach.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biology15040305/s1>. Figure S1: Dose-Response Curves for Hes and DOX in WERI-Rb-1 Cells (Log-Transformed Concentration Axis).

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Abbreviations

RB	Retinoblastoma
DOX	Doxorubicin
HES	Hesperidin
ECM	Extracellular Matrix
MMP	Matrix Metalloproteinase
α -SMA	Alpha Smooth Muscle Actin
TIMP	Tissue Inhibitor of Metalloproteinases
Bax	Bcl-2-associated X protein

Bcl-2	B-cell lymphoma 2
WERI-Rb-1	Human retinoblastoma cell line
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IC ₅₀	Half Maximal Inhibitory Concentration
PI	Propidium Iodide
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
GO	Gene Ontology
BP	Biological Process
PPI	Protein–Protein Interaction
KEGG	Kyoto Encyclopedia of Genes and Genomes
CI	Combination Index
ANOVA	Analysis of Variance
SD	Standard Deviation

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