

Regorafenib-Induced Stress Response Alters the Bioenergetic Profile of Osteosarcoma Cells and Modulates Gene Expression Associated with Metabolic regulation-a Potential Mechanism of Osteosarcoma Treatment-Related Adaptation

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Purpose: Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. Despite advances in surgery and multiagent chemotherapy, outcomes remain poor for patients with metastatic or recurrent disease. Regorafenib, an oral multikinase inhibitor targeting pathways involved in tumor proliferation, angiogenesis, and metastasis, has shown clinical promise in osteosarcoma; however, its mechanisms of action are not fully understood.

Methods: We investigated the effects of regorafenib in a human osteosarcoma cell line (MG63) and a patient-derived primary cell line (APR1). Cells were treated at their respective IC₅₀ concentrations (26 μM for MG63 and 42 μM for APR1). Regorafenib-induced changes in cell viability, apoptosis, migration, invasion, and cell-cycle distribution were assessed. Mitochondrial bioenergetics were evaluated using Seahorse XF technology. Molecular effects were analyzed by assessing gene and protein expression related to cancer-associated pathways and by RT² Profiler PCR arrays targeting key oncogenic genes.

Results: Regorafenib significantly reduced cell viability, migration, and invasion and induced apoptosis in both cell models. MG63 cells exhibited greater sensitivity, reflected by a lower IC₅₀ value. Cell-cycle analysis revealed G2/M arrest in MG63 cells and G0/G1 accumulation in APR1 cells, indicating distinct adaptive responses. At the molecular level, regorafenib modulated the expression of apoptotic regulators and noncoding RNAs. Suppression of the PI3K/AKT/mTOR pathway occurred predominantly via inhibition of protein phosphorylation rather than transcriptional downregulation. Metabolic analyses showed reduced ATP production through both oxidative phosphorylation and glycolysis in MG63 cells, while APR1 cells preserved glycolytic activity. Transcriptomic profiling revealed differential regulation of genes associated with oncogenesis, hypoxia, DNA damage response, and angiogenesis.

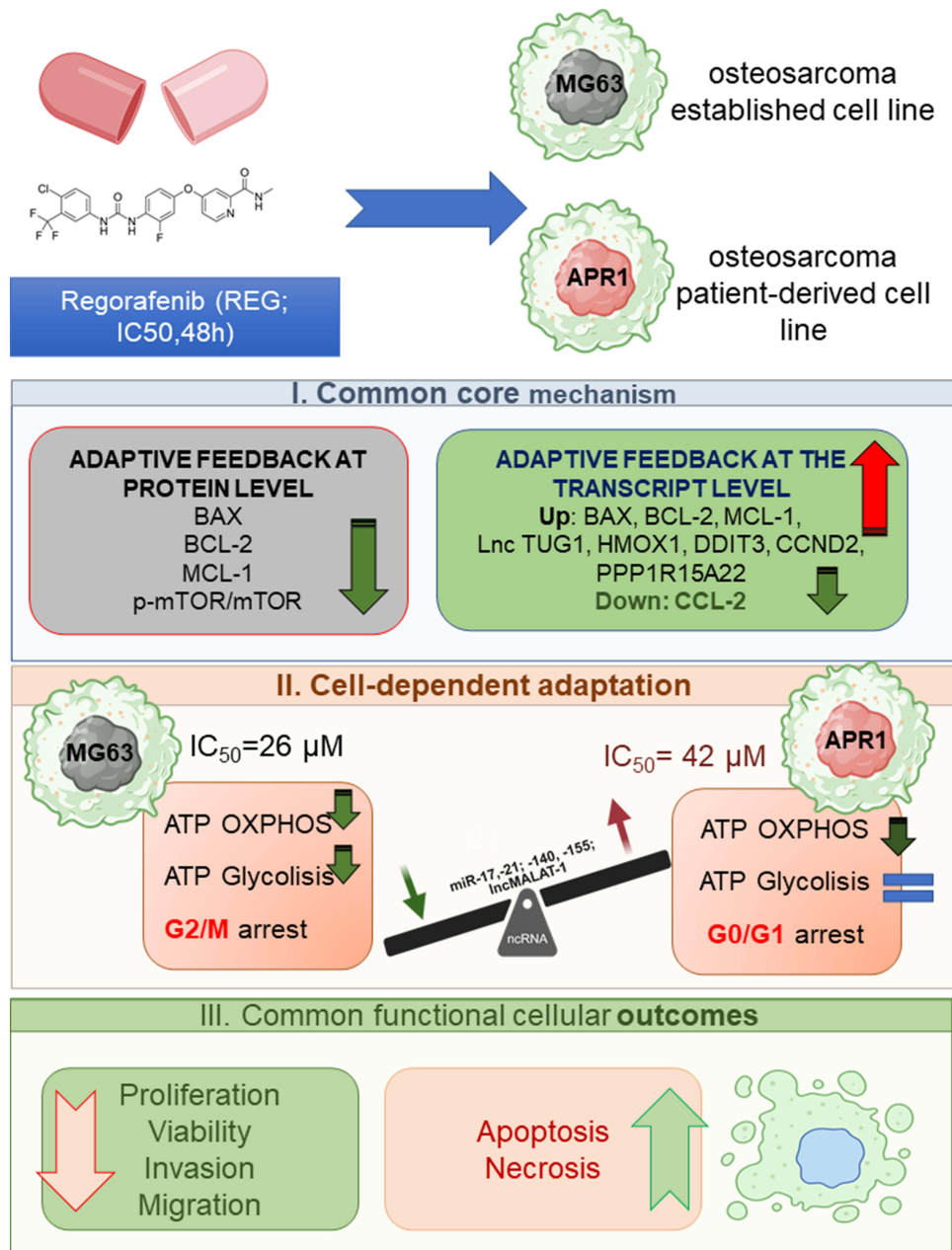
Conclusion: These findings demonstrate the multifaceted antitumor activity of regorafenib in osteosarcoma and highlight cell context-dependent responses. The study provides new insights into molecular and metabolic mechanisms underlying regorafenib activity and supports further investigation of this agent in osteosarcoma therapy.

Keywords: osteosarcoma, regorafenib, cell viability, ATP metabolism, bioenergetic profile, gene expression profiling

Introduction

Osteosarcoma is the most common primary malignant bone tumor, with the highest incidence occurring in children and adolescents. The current standard of treatment involves surgical resection combined with multiagent chemotherapy, typically including doxorubicin, methotrexate,¹ cisplatin, ifosfamide² and etoposide.³ Advances in therapeutic strategies

Graphical Abstract



have significantly improved the 5-year survival rate for patients with localized osteosarcoma, which now exceeds 70%.⁴ However, the prognosis remains poor in cases of metastasis, chemoresistance, or disease recurrence. Unfortunately, even worse prognoses are observed for patients with metastatic osteosarcoma; here, the 5-year survival rate remains less than 30%.⁵ As evidenced by current statistics, osteosarcoma remains a significant therapeutic challenge, underscoring the need for more precise and effective treatment strategies.

The expression of multiple tyrosine kinase receptors (RTKs) in osteosarcoma plays a critical role in promoting tumor cell proliferation, differentiation, and invasion. Tyrosine kinase inhibitors (TKIs) exert their therapeutic effects by selectively inhibiting ligand binding to RTKs, thereby disrupting the activation of essential intracellular signaling

cascades associated with MAPK, PI3K, and Src family proteins. Several studies have reported the potential of TKIs to prolong progression-free survival in patients with bone sarcomas.^{6,7}

Among the TKIs investigated for their antitumor efficacy in bone sarcomas, regorafenib has gained particular attention because of its broad spectrum of targets and multifaceted mechanism of action.^{7,8}

Regorafenib is an innovative and potent small-molecule multikinase inhibitor that exerts antitumor effects by modulating a wide range of dysregulated signaling pathways involved in tumor progression. It interferes with key pathological processes, such as oncogenic transformation, tumor-induced angiogenesis, metastasis, and the tumor immune response. Regorafenib also has a wide range of antitumor activities. It inhibits key kinases such as VEGFR1–3, RET, KIT, PDGFR- α/β , FGFR1/2, RAF-1, BRAF, and others that are critically implicated in cancer progression.⁹

The significant clinical and preclinical efficacy of regorafenib has been shown for hepatocellular carcinoma,^{10,11} metastatic colorectal cancer,¹² and gastrointestinal stromal tumors.¹³ Given its broad-spectrum kinase inhibition ability and ability to disrupt multiple oncogenic pathways, regorafenib is also increasingly recognized as a promising therapeutic candidate in the management of osteosarcoma. Several systemic treatments have been evaluated for patients with relapsed or treatment-resistant osteosarcoma. Regorafenib is the only drug strongly recommended by the National Comprehensive Cancer Network (NCCN) as a second-line therapy, supported by high-level clinical evidence (category 1). Other options, such as high-dose ifosfamide with etoposide or sorafenib, are also suggested for second-line use, although the supporting evidence for these therapies is less conclusive.¹⁴ Regorafenib has demonstrated beneficial effects in slowing disease progression in adult patients with progressive metastatic osteosarcoma following unsuccessful conventional chemotherapy.^{15,16}

In the randomized, placebo-controlled Phase II REGOBONE trial, Duffaud et al¹⁵ showed that regorafenib administered at 160 mg/day for 21 of 28 days in patients with relapsed metastatic osteosarcoma produced a substantially higher 8-week non-progression compared with placebo. The safety profile, while manageable, includes considerable toxicity (e.g., hypertension and hand–foot skin reaction among the most frequent grade ≥ 3 events). Moreover, frequent dose interruptions/reductions have been reported, indicating that the long-term tolerability of continuous regorafenib exposure may be limited.

In addition, a multicenter phase II study by Davis et al¹⁶ reported improved progression-free survival and disease stabilization in adults with recurrent metastatic osteosarcoma treated with regorafenib, confirming its antitumor activity across independent cohorts. In both trials, regorafenib was administered at a standard oral dose of 160 mg daily (3 weeks on/1 week off).

A recent systematic review not only highlighted regorafenib's potential to delay disease progression in metastatic or recurrent bone sarcomas, but also, in light of the higher frequency of adverse events noted, underscored the need to better define its therapeutic window through optimised dosing strategies and rational combination approaches.⁶

All these clinical observations highlight the importance of understanding cellular adaptation programs that enable osteosarcoma cells to withstand regorafenib pressure and ultimately escape treatment. Although the exact mechanism of regorafenib action in osteosarcoma remains unclear, it is thought to involve interference with angiogenic signaling and modulation of the tumor microenvironment (TME). Reprogramming the TME represents a promising therapeutic strategy for cancer treatment. The inhibition of angiogenesis plays a crucial role in modulating macrophage polarization, hindering tumor progression, and enhancing drug delivery within tumor tissue.⁸

Insights into the molecular mechanism of action of regorafenib are limited and are primarily derived from studies utilizing the U2 OS cell line, a well-established model for human osteosarcoma. According to findings by Pan et al¹⁷ regorafenib induces robust proapoptotic effects. The apoptotic cascade triggered by regorafenib involves the activation of caspases 3 and 8, key executioners of programmed cell death. Notably, regorafenib also enhances the expression of FAS and FASL, which are markers indicative of extrinsic, receptor-mediated apoptotic signaling. This proapoptotic effect of regorafenib was also associated with the downregulation of crucial antiapoptotic proteins, including XIAP, c-FLIP, and MCL-1, highlighting the capacity of regorafenib to shift the cellular balance in favor of apoptosis.

In turn, the reduced proliferative activity of U2 OS cells was reflected by a significant increase in the subG1 population and a noticeable decrease in the proportion of cells in the S phase. Moreover, Pan et al¹⁷ demonstrated that regorafenib treatment resulted in decreased expression and phosphorylation of AKT and ERK, key components of signaling pathways involved in cell proliferation and survival. In addition to its effects on U2 OS cell viability, Pan et al¹⁷ demonstrated that regorafenib modulates the invasive properties of osteosarcoma cells. This was marked by a reduction

in the expression of VEGF and MMP-9, which are critical molecules for angiogenesis and extracellular matrix degradation. Importantly, the molecular and phenotypic effects of regorafenib are dose dependent, underscoring the importance of optimal therapeutic dosing. In studies utilizing a murine model bearing U2 OS xenografts, the administration of regorafenib at a dose of 10 mg/kg resulted in a pronounced decrease in tumor volume. Notably, this antitumor effect occurred without any detectable hepatotoxicity, suggesting a favorable therapeutic index and potential for clinical application. Together, findings by Pan et al¹⁷ highlight the multifaceted antitumor potential of regorafenib in osteosarcoma and support its continued investigation as a therapeutic agent in both preclinical and clinical settings.

In the present study, we comprehensively investigated the biological effects of regorafenib at its IC₅₀ on osteosarcoma cells using both the well-established MG63 cell line and the primary osteosarcoma cell line APR1. We employed two complementary osteosarcoma models to capture whether key phenotypic and molecular responses are shared across distinct osteosarcoma backgrounds rather than being cell line-specific.

MG-63 cells are among the most widely used *in vitro* osteosarcoma models and provide a well-characterised platform for interrogating canonical pathways of drug response and resistance. Their use also enables direct comparison with the existing literature and enhances reproducibility across laboratories.¹⁸

In contrast, application of primary patient-derived cells, such as APR1 cell line established in our lab from patient samples provides a clinically relevant model. The primary cell lines retain more of the genetic heterogeneity and phenotypic variability observed in clinical tumors, making them more representative of patient-specific disease biology and better suited to identifying adaptive cellular responses, potentially not fully captured by long-term established cell lines.¹⁹

Our primary focus was to elucidate the role of regorafenib in modulating key cancer-related processes, such as viability, proliferation, and metastatic potential. To better understand the mode of action of regorafenib, we also investigated its impact on the PI3K/AKT/mTOR signaling pathway, a crucial regulatory axis frequently implicated in osteosarcoma pathogenesis and progression.

Notably, for the first time, we assessed the impact of regorafenib on bioenergetic functions, focusing specifically on real-time ATP production, to determine the metabolic alterations induced by the drug. The bioenergetic reprogramming is recognised as a key adaptive mechanism underlying osteosarcoma progression and therapy resistance. Osteosarcoma cells display pronounced metabolic plasticity, including differential reliance on glycolysis and oxidative phosphorylation, which is closely linked to oncogenic signalling pathways such as PI3K/AKT/mTOR and to cellular stress responses induced by anticancer agents. Importantly, these metabolic adaptations integrate multiple resistance mechanisms and may determine treatment outcome more effectively than single-gene alterations. Accordingly, in this study we profiled cellular energy production under regorafenib-induced pharmacological stress to capture potential treatment-driven bioenergetic adaptation and identify metabolic liabilities relevant to drug response and resistance.^{20,21}

Additionally, we conducted a high-throughput gene expression analysis of genes closely associated with cancer progression, thereby identifying potential molecular mechanisms underlying the anticancer effects of regorafenib.

In the present study, we hypothesised that regorafenib alters key cellular features associated with osteosarcoma malignant potential, including proliferation, viability, migration and invasion and may elicit cell-dependent molecular adaptation. Specifically, we posited that regorafenib exposure modulates progression-associated markers and the PI3K/AKT/mTOR signalling axis and is accompanied by coordinated transcriptomic changes in metabolism- and stress-adaptation-related gene programmes, including noncoding RNA patterns, consistent with an adaptive response under pharmacological pressure.

Our results provide novel insights into the multifaceted activity of regorafenib, highlighting its therapeutic potential not only in modulating the viability and invasiveness of osteosarcoma cells but also in altering ATP synthesis pathways and the expression of genes involved in tumor adaptation and cellular reprogramming.

Materials and Methods

Cell Culture

Regorafenib activity was analyzed *in vitro* using a well-established human osteosarcoma cell line, MG63 (ATCC; CRL-1427), and primary APR1 cells established from patient tissue. The MG63 and APR1 cells used for the assays were at passages 6 and 5,



respectively. The cells were verified for Mycoplasma contamination by IDEXX BioAnalytics (Germany). The biopsy material used for isolation of the APR1 cell line was derived from the Institute of Mother and Child in Warsaw. To confirm the osteosarcoma diagnosis, a histological analysis of the biopsy was conducted. Written consent for the use of biopsy material for cell isolation was obtained from the patient's parents. This study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Local Institutional Review Board at the Institute of Mother and Child (no. 53/2021). The cell isolation protocol has been described in detail elsewhere.²² The MG63 cell lines were cultured in complete growth medium (CGM) composed of minimum essential medium Eagle (EMEM, Sigma Aldrich/Merck, Poznań, Poland) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S), whereas the APR1 cell line was maintained in CGM supplemented with Dulbecco's modified Eagle's medium low glucose (DMEM LG, 1000 mg/L glucose, Sigma Aldrich/Merck, Poznań, Poland) supplemented with 15% FBS and 1% P/S. The cultures were kept under constant sterile conditions in a CO₂ incubator at 37°C with 95% humidity. The growth medium was replaced every 2–3 days, and the cells were passaged upon reaching 70% confluence via stable-cell trypsin solution (Sigma Aldrich/Merck, Poznań, Poland).

Preparation of Regorafenib for in vitro Assays

Regorafenib was purchased from MedChemExpress (New Jersey, USA) and dissolved in sterile DMSO (Sigma Aldrich/Merck, Poznań, Poland) to a final concentration of 1 mM. Before use, it was filtered through a 0.22 µm syringe filter.

Screening Assay – Assessment of Regorafenib-Induced Cytotoxicity, Metabolic Effects, and Determination of the IC₅₀ Value

An MTS assay (Cell Proliferation, Colorimetric, Abcam, Cambridge, UK) was used to evaluate the cytotoxicity and metabolic effects of regorafenib on osteosarcoma cell lines. The cells were seeded in 96-well plates at a density of 1×10^4 (MG63) and 5×10^3 cells per well (APR1) in 200 µL of CGM. The inoculum was adjusted to 70% confluency after 24 hours. Following cell adhesion, a screening assay was performed, and regorafenib was added to the cultures at concentrations of 2.5, 5, 10, 25, 50, 100, 250, 500, 750 and 1000 µM. Cytotoxicity was evaluated after 48 hours of exposure to regorafenib via the MTS assay. The procedure was performed following the manufacturer's instructions. After the reagents were added, the cultures were incubated for 2 hours at 37°C in a CO₂ incubator. The experiments included control (vehicle) and blank (samples containing medium and MTS reagent without cells). For the MTS assay, each biological replicate was run on a balanced well layout and plate-matched normalisation to minimise edge, positional, and batch effects. The absorbance was subsequently measured at 490 nm via a Spark 10 M plate reader (Tecan, Austria). Background absorbance values were subtracted from all measurements. The IC₅₀ values were calculated using the AAT Bioquest IC₅₀ Calculator (Quest Graph™ IC₅₀ Calculator. AAT Bioquest. <https://www.aatbio.com/tools/ic50-calculator>) and verified in GraphPad software (version 9.5.1; San Diego, CA, USA) by applying nonlinear regression using a four-parameter logistic model (log[inhibitor] vs. response, variable slope). The IC₅₀ estimates are reported with 95% confidence intervals.

Experimental Cultures

For functional assays, cells were seeded at a density of 1.6×10^4 cells/cm² for APR1 and 3.2×10^4 cells/cm² for MG63, which allowed the cultures to reach approximately 70% confluency after 24 hours. The experimental cultures were treated for 48 hours with regorafenib at concentrations corresponding to the IC₅₀ values specific to each cell line: 26 µM for MG63 and 42 µM for APR1. The control group was treated with an equivalent volume of sterile DMSO to replicate the experimental conditions (vehicle control). The corresponding final concentrations of DMSO were 0.26% (v/v) for MG63 and 0.42% (v/v) for APR1. After incubation with regorafenib, the cellular activity of the osteosarcoma cell lines MG63 and APR1 was monitored at various levels.

Cell Morphology and Growth Pattern

To evaluate the influence of regorafenib on osteosarcoma cell morphology and growth architecture, MG63 and APR1 cells were seeded on microscope slides with a diameter of 12 mm placed within 24-well plates. After treatment, the

mitochondria in the live cultures were stained with MitoRED dye (Sigma–Aldrich/Merck, Poznań, Poland) prepared in CGM at a 1:1000 dilution. The cells were then incubated for 30 minutes at 37°C in a 5% CO₂ incubator. After staining, the cultures were fixed in ice-cold 4% paraformaldehyde (PFA; Sigma–Aldrich/Merck Life Science Sp. z o.o., Poznań, Poland) for 30 minutes at room temperature. After fixation, the samples were washed three times with PBS. Cell membranes were permeabilized with a 0.1% Triton X-100 solution prepared in PBS, and cultures were maintained in permeabilization solution for 15 minutes at room temperature. Briefly, actin filaments were stained with atto-488-labeled phalloidin diluted 1:800 in PBS. The cells were incubated with the dye for 40 minutes at room temperature in the dark. Nuclear counterstaining was performed via a DAPI-containing mounting medium (ProLong™ Diamond Antifade Mountant with DAPI; Thermo Fisher Scientific, Warsaw, Poland). The staining protocols were comprehensively detailed in our previous studies.²³

Migratory Capacity

The effect of regorafenib on the migratory potential of osteosarcoma cell lines was evaluated using wound healing and invasion assays applying previously published protocols.^{24,25} To assess treatment-induced changes in cell behavior, cells were trypsinized after exposure to regorafenib, resuspended in CGM, and seeded into 24-well plates. For the wound healing assay, cell cultures were grown to 90% confluence. A scratch was created via a 200-μL pipette tip, followed by a 24-hour incubation. The cultures were then fixed with 4% PFA and stained with a 2% pararosaniline solution. Imaging was performed using an Axiocam 208 color (Zeiss) camera. Wound closure was quantified by capturing phase-contrast images at defined time points (0 h and 24 h) using the same microscope settings and fields of view. The wound area was measured using ImageJ software, and the healing process was expressed as the gap width [μm] of wound closure relative to the initial wound area at time 0.

To determine the influence of regorafenib on the invasiveness of osteosarcoma cells, the cells were harvested and resuspended in serum-free medium after the test. The cells were seeded into the upper chamber of a Transwell insert (ThinCerts, Greiner Bio-One, Kremsmünster, Austria), while the lower chamber was filled with complete medium. The cells were incubated at 37°C for 48 hours. Nonmigrated cells were removed, whereas the invading cells remaining on the membrane were fixed with 4% PFA and stained with 2% pararosaniline. The lower surfaces of the membranes were imaged and analyzed under a light microscope at 40x and 100x magnification using an Axiocam 208 color (Zeiss) camera. Quantitative analysis was performed by counting cells in selected microscopic fields per insert using ImageJ software with the Pixel Counter plugin (version 1.6.0, U. S. National Institutes of Health, Bethesda, MD, USA).²⁶ Results were expressed as the percentage of migrated cells per field and normalised to the corresponding control conditions.

Viability and Cell Cycle Progression

Following incubation with regorafenib, cells (1×10^6) were harvested and washed with cold phosphate-buffered saline (PBS) to evaluate the impact of the drug on viability and cell cycle progression.

The apoptosis profile was evaluated using Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry (Invitrogen Life Technologies, Warsaw, Poland) according to the manufacturer's protocol. The washed cells were resuspended in 100 μL of 1X annexin-binding buffer. To stain apoptotic and necrotic cells, 5 μL of Alexa Fluor™ 488 Annexin V and 1 μL of a 100 μg/mL propidium iodide (PI) working solution were added to the cell suspension. The cells were incubated at room temperature for 15 minutes, after which 400 μL of 1X annexin-binding buffer was added. The samples were then mixed and kept on ice. The stained cells were analyzed with a CytoFLEX flow cytometer (Beckman Coulter, CA, USA), and the fluorescence emission at 530 nm and 575 nm was measured via 488-nm excitation to distinguish live, dead and apoptotic cells. The acquired data were analyzed using CytExpert 2.4 software (Beckman Coulter, CA, USA).

To analyze DNA content distribution, FxCycle™ PI/RNase Staining Solution (Invitrogen Life Technologies, Warsaw, Poland) was used. The analysis was performed according to the manufacturer's instructions. After trypsinization, the cells were fixed in 70% ethanol and incubated at 4°C for 24 hours. The cells were washed to ensure complete removal of the fixative before staining. Next, the samples were centrifuged ($500 \times g$ for 5 min at 4°C). To each sample, 0.5 mL of FxCycle™ PI/RNase Staining Solution was added, and gently mixed to ensure uniform staining. The samples were

stained for 30 minutes at room temperature, protected from light. Following incubation, the samples were analyzed without washing using 488-nm excitation, and fluorescence emission was measured via a 585/42 nm bandpass filter.

For each condition, 30,000 single-cell events were acquired. Doublets and aggregates were excluded using FSC-A versus FSC-H and/or FSC-W discrimination. The gating strategy included initial exclusion of debris based on FSC/SSC parameters, followed by singlet gating and, where applicable, a live-cell gate, with subsequent quadrant or region gating applied for apoptosis or cell-cycle analysis. Gates were defined using appropriate controls and applied consistently across samples. For apoptosis analysis, gate placement was based on unstained cells, and fluorescence compensation was verified using single-stained compensation controls; where applicable, an apoptosis-positive control was included. For cell-cycle analysis, gates were set on singlet populations, and DNA-content histograms were analyzed using a standard cell-cycle model with untreated control samples serving as the reference.

Effect of Regorafenib on Bioenergetic Profile of Osteosarcoma Cell Lines

To characterize cellular metabolism and bioenergetic characteristics of the cells treated with regorafenib, the rate of adenosine triphosphate (ATP) production was measured using the Seahorse XF Real-Time ATP Rate Assay (Agilent Technologies, Santa Clara, CA). The assessments were performed with a Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer's guidelines. Before analysis, CGM was replaced with the assay media, which consisted of Seahorse XF DMEM (pH 7.4) supplemented with 10 mM XF glucose, 1 mM XF pyruvate, and 2 mM XF glutamine. The cells were then incubated in a non-CO₂ incubator for one hour. Next, a sensor cartridge was loaded with oligomycin at a final concentration of 1.5 μ M and a combination of rotenone and antimycin A at a final concentration of 0.5 μ M. The acquired Seahorse data were analyzed using Wave software (Agilent Technologies, Santa Clara, CA). ATP production rate data were normalized to the cell number per well. Normalization parameters were entered using the Normalize module in Seahorse Wave software prior to data export, and the normalized values were subsequently used for calculations and graphical presentation in the Seahorse Report Generator.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) for the Detection of mRNAs and miRNAs

After the experiment, the expression of the selected genes was analyzed. Total RNA was extracted using 1 mL of TRI Reagent (Sigma Aldrich/Merck, Poznań, Poland) following the manufacturer's protocol. The isolated RNA was diluted in nuclease-free water (Sigma Aldrich/Merck, Poznań, Poland), and its concentration and purity were assessed spectrophotometrically at 260 and 280 nm using a DS-11 Fx spectrophotometer (Denovix, Wilmington, DE, USA). To ensure RNA integrity, DNase I digestion using PrecisionDNase kit (PrimerDesign, BLIRT S.A., Gdańsk, Poland) was performed prior to reverse transcription; 500 ng of total RNA was used to detect mRNAs, and 375 ng was used to detect noncoding RNAs. cDNA synthesis was carried out with the Tetro cDNA Synthesis Kit (Bioline Reagents Limited, London, UK) or Mir-X™ miRNA First-Strand Synthesis Kit (Takara Clontech Laboratories, Biokom, Poznań, Poland) according to the manufacturer's instructions in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The synthesized cDNA was used for RT-qPCR analysis with the SensiFAST SYBR® and Fluorescein Kit (Bioline Reagents Ltd., London, UK). Each reaction had a final volume of 10 μ L, comprising 1 μ L of cDNA, 5 μ L of Master Mix, and primers at a final concentration of 400 nM for mRNA and 200 nM for miRNA. Quantitative PCR was conducted via the CFX Opus 384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The cycling conditions included initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at the primer-specific temperature for 15 seconds, and elongation at 72°C for 15 seconds (elongation step only for mRNA detection). Gene expression analysis was normalized to the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or snU6 (for miRNA) and quantified using the RQ_{MAX} method, with the results presented on a logarithmic scale. Primers were designed using the NCBI Nucleotide database and Primer-BLAST (Pick Primers),²⁷ annealing temperatures were experimentally optimized annealing temperatures and specificity of assays was confirmed by melt-curve analysis and with both no-template (NTC) and no-reverse transcription (NoRT) controls included to rule out contamination and genomic DNA carryover. The sequences of primers used in the study are listed in [Table S1](#).

Protein Expression

Following cell culture under experimental conditions, cells were lysed in ice-cold RIPA buffer (Sigma Aldrich/Merck, Poznań, Poland) supplemented with 1% phosphatase and protease inhibitors (Thermo Fisher Scientific, Warsaw, Poland). Protein concentration was determined via a Bicinchoninic Acid Assay Kit (BCA; Thermo Fisher Scientific, Warsaw, Poland). A total of 10 µg of protein per sample was mixed with Laemmli loading buffer (Bio-Rad, Hercules, CA, USA) and denatured at 95°C for 5 minutes. The prepared samples, along with the BLUeye Prestained Protein Ladder (Sigma Aldrich/Merck, Poznań, Poland), were separated using 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 100 V for 90 minutes. Following electrophoresis, proteins were transferred onto a PVDF membrane using 1× Tris-glycine buffer (Bio-Rad, Hercules, CA, USA) via the Mini Trans-Blot® system (Bio-Rad, Hercules, CA, USA) at 100 V for 60 minutes. The membranes were blocked for 1 hour in 3% BSA (Sigma Aldrich/Merck, Poznań, Poland) prepared in Tris-buffered saline with Tween 20 (TBST, Bio-Rad, Hercules, CA, USA). The membranes were subsequently incubated overnight at 4°C with shaking in the presence of specific primary antibodies, as listed in [Table S2](#). After incubation, membranes were washed five times for 5 minutes each with TBST buffer, followed by a 60-minute incubation at room temperature with an HRP-conjugated secondary antibody. All antibodies were diluted in 3% BSA (Sigma Aldrich/Merck, Poznań, Poland) in TBST buffer. The membranes were then washed as previously described and analyzed via the Bio-Rad ChemiDoc™ XRS system (Bio-Rad, Hercules, CA, USA) with Westar Hypernova Substrate (Cynagen, Bologna, Italy). Signal intensity was quantified by densitometric analysis in Image Lab™ software (version 6.1; Bio-Rad: Hercules, CA, USA; 2020). For each sample, the intensity of the target protein band was normalized to the corresponding β-actin band.

Cancer-Related Gene Expression Analysis Using RT² Profiler PCR Arrays

The Human Cancer PathwayFinder RT² Profiler PCR Array (PAHS-033Z, QIAGEN, Hilden, Germany) was used to evaluate the expression of 84 genes associated with nine biological pathways relevant to cellular transformation and cancer development. The procedure was performed according to the manufacturer's instructions. The protocol began with the synthesis of first-strand cDNA from 500 ng of total RNA using the RT² First Strand Kit (QIAGEN, Hilden, Germany). Initially, the genomic DNA elimination mixture was prepared for each RNA sample according to the manufacturer's protocol. After gentle mixing, the samples were briefly centrifuged, incubated at 42°C for 5 minutes, and then immediately placed on ice for at least 1 minute. Next, the reverse transcription mixture was prepared, and 10 µL of this mix was added to each tube containing 10 µL of the genomic DNA elimination mixture. The samples were incubated at 42°C for exactly 15 minutes, followed by immediate inactivation of the reverse transcriptase at 95°C for 5 minutes. The samples were incubated in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Subsequently, 91 µL of RNase-free water was added to each reaction and mixed thoroughly by pipetting. The resulting cDNA samples were kept on ice before real-time PCR was performed. A targeted analysis of oncogenesis-related gene expression was conducted using the RT² Profiler™ PCR Array Human Cancer PathwayFinder™ (QIAGEN, Hilden, Germany). For each reaction, the cDNA template, RNase-free water, and RT² SYBR® Green Mastermix (QIAGEN, Hilden, Germany) were mixed following the manufacturer's guidelines and dispensed into the PCR array plates in a final volume of 10 µL per well. Amplification was carried out via the CFX Opus 384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions included initial activation of the HotStart DNA Taq Polymerase at 95°C for 10 minutes, followed by 40 cycles of fluorescence data collection at 95°C for 15 seconds and at 60°C for 60 seconds. The ramp rate between 95°C and 60°C was set at 1°C/sec. To confirm the specificity of the PCRs, dissociation curve analysis was performed with the following settings: 95°C for 1 minute, 65°C for 2 minutes (optics off), and a gradual increase from 65°C to 95°C at 2°C/min (optics on). Gene expression levels were normalized to those of the reference genes ACTB (β-actin), B2M (β-2-microglobulin), RPLP0 (ribosomal protein, large, P0), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and were calculated using the 2^{−(ΔΔCt)} method. The obtained data were analyzed via the GeneGlobe Data Analysis Center (QIAGEN, Hilden, Germany). Differentially expressed genes were identified based on a combined threshold of $p < 0.05$ and a predefined fold-change cut-off, in accordance with the manufacturer's recommendations for exploratory RT² array analyses.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, CA, USA). Normality was assessed using the Shapiro–Wilk test, and homogeneity of variances using Levene’s test (with Brown–Forsythe as a robustness check when indicated). When assumptions for parametric testing were not met, non-parametric alternatives were applied. Depending on the dataset, either Student’s *t* test or one-way ANOVA followed by Šidák’s multiple-comparisons test (for pre-specified contrasts) was applied. All experiments were performed using at least two independent biological replicates; the number of technical replicates varied between assays and is specified in the figure legends. Sample size was calculated using Stein’s two-stage procedure.²⁸ A pilot set of independent replicates was used to estimate variability, and the final number of replicates was adjusted to ensure the required minimum number of replicates. The results were considered statistically significant at a *p* value less than 0.05. Statistical significance was indicated using asterisks as follows: **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Differences that were not statistically significant are marked as “ns”.

Results

The IC₅₀ of Regorafenib Differs Between the Well-Established Osteosarcoma Cell Line MG63 and the Primary Cell Line APR1

The IC₅₀ value for regorafenib was determined in both cell lines with the MTS assay (Figure 1). The drug was tested at concentrations of 2.5, 5, 10, 25, 50, 100, 250, 500, 750, and 1000 μM, and the cytotoxicity as well as metabolism of the cells were evaluated after 48 hours. The IC₅₀ for regorafenib in MG63 cells was determined to be 26 ± 3 μM (Figure 1a), whereas in APR1 cells, it was higher and more variable, at 42 ± 12 μM (Figure 1b). Furthermore, compared with APR1 cells, MG63 cells exhibited a more reproducible response to treatment, which is consistent with the expected heterogeneity of primary cell populations. Analysis of the growth curves and IC₅₀ estimates suggests that, under the tested conditions, MG63 cells exhibit higher sensitivity to the drug than primary APR1 cells.

IC₅₀ of Regorafenib Affects the Growth Pattern of Osteosarcoma Cells

The morphology of the osteosarcoma cells was assessed via epifluorescence microscopy following the staining of key subcellular structures: the nuclei were stained with DAPI, the actin cytoskeleton was stained with phalloidin–Atto 488, and the mitochondria were stained with MitoRed (Figure 2). In control cultures treated with vehicle (DMSO), the cells maintained a typical morphology. Both MG63 and APR-1 cells are heterogeneous in morphotype. MG63 cells exhibited a predominantly fibroblast-like appearance, with the presence of multinucleated giant cells observed under control conditions. In contrast, APR-1 cells were mainly spindle shaped and bipolar.

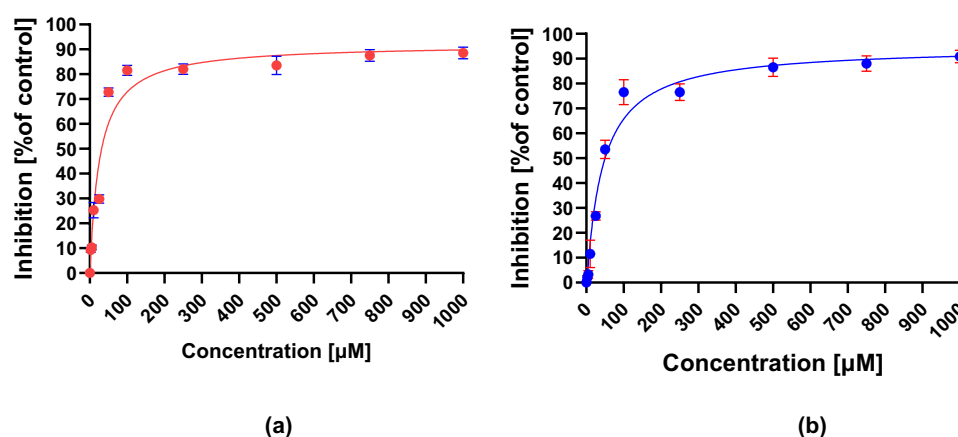


Figure 1 Representative dose–response curves used to determine the IC₅₀ values for a well-established osteosarcoma cell line, MG63 (REG IC₅₀ 26 ± 3 μM) (a), and primary cells, APR1 (REG IC₅₀ 42 ± 12 μM) (b). Data are presented as mean $\pm$ SD from *n* = 3 biological replicates, each performed in 4 technical replicates.

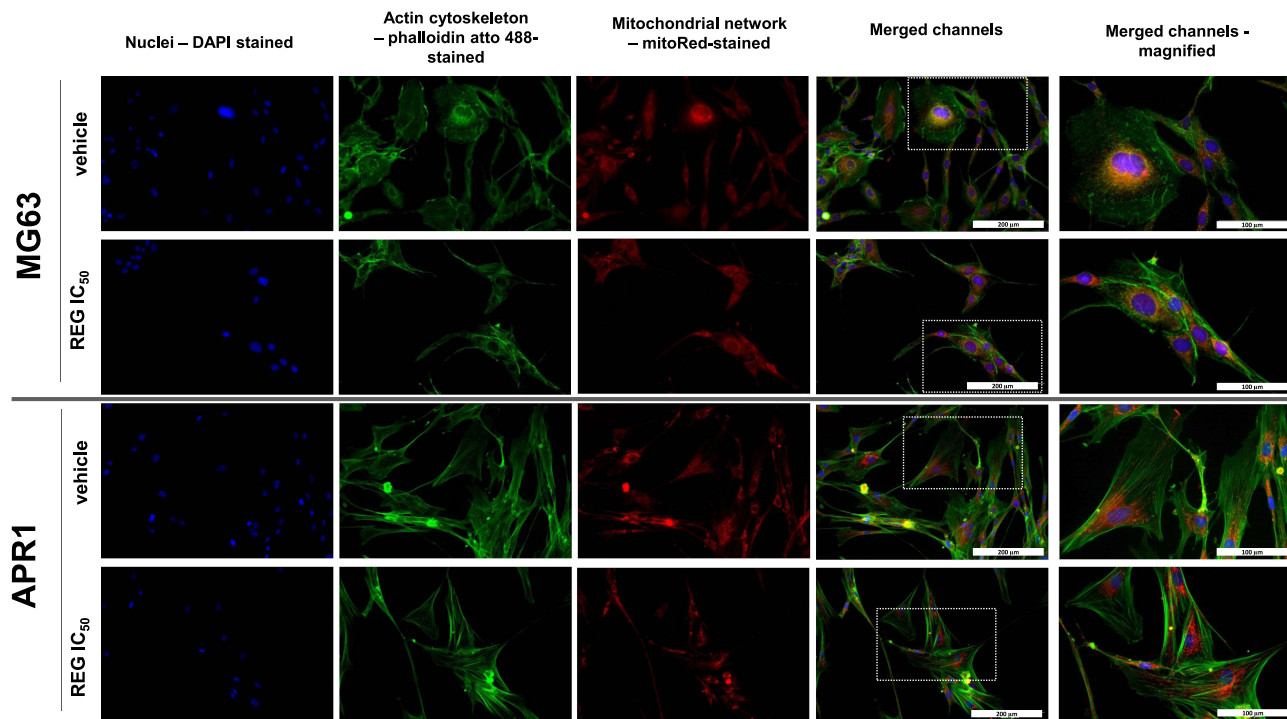


Figure 2 Visualization of the morphology of MG63 and APR1 osteosarcoma cells by epifluorescence microscopy under control (DMSO-treated) and regorafenib-treated (IC₅₀) conditions. Key cellular structures were visualized via fluorescent dyes: the nuclei were stained with DAPI (blue), the actin cytoskeleton was stained with phalloidin Atto-488 (green), and the mitochondrial network was stained with mitoRed (red). Magnification 100x, scale bar: 200 μ m; 200x, scale bar: 100 μ m.

Exposure to regorafenib altered the growth dynamics of the osteosarcoma cultures, leading to a noticeable reduction in confluency. This observation is consistent with the established IC₅₀ values, confirming the efficacy of the drug under the tested conditions.

Regorafenib Has Differential Effects on Migration and Invasion in Osteosarcoma Cell Models

Regorafenib treatment impaired the migratory and proliferative capacities of both MG63 and APR1 osteosarcoma cells, as demonstrated by the wound healing assay (Figure 3). Despite the inhibitory effect of regorafenib, APR1 cells consistently exhibited a greater wound closure rate than MG63 cells (Figure 3a and b), both in the control and treated groups (Figure 3c).

Regorafenib treatment at the IC₅₀ also significantly suppressed the invasive capacity of both osteosarcoma cell lines, confirming the drug's potential to limit metastatic progression by impairing cell motility and invasive behavior (Figure 4a and b).

Regorafenib Promotes Apoptosis and Modulates Cell Cycle Progression in Osteosarcoma Cells in a Manner That Reflects Their Resistance Phenotype

The influence of regorafenib on cell viability was assessed on the cell death profile determined by Annexin V/PI staining (Figure 5a–d). The analysis revealed that regorafenib reduced cell viability (Figure 5b) by inducing cell death through multiple pathways, as evidenced by a significantly increased proportion of early (Figure 5d) and late apoptotic cells (Figure 5e), as well as an increase in necrotic cell populations (Figure 5c). The analysis of the impact of regorafenib on cell viability was complemented by the assessment of apoptosis-related markers, which were measured at the mRNA and protein levels (Figure 6). At the mRNA level, we noted significant upregulation of both the proapoptotic gene BAX (Figure 6a) and the antiapoptotic genes BCL2 (Figure 6c) and MCL1 (Figure 6h) in MG63 and APR1 cells following treatment with regorafenib at the IC₅₀.

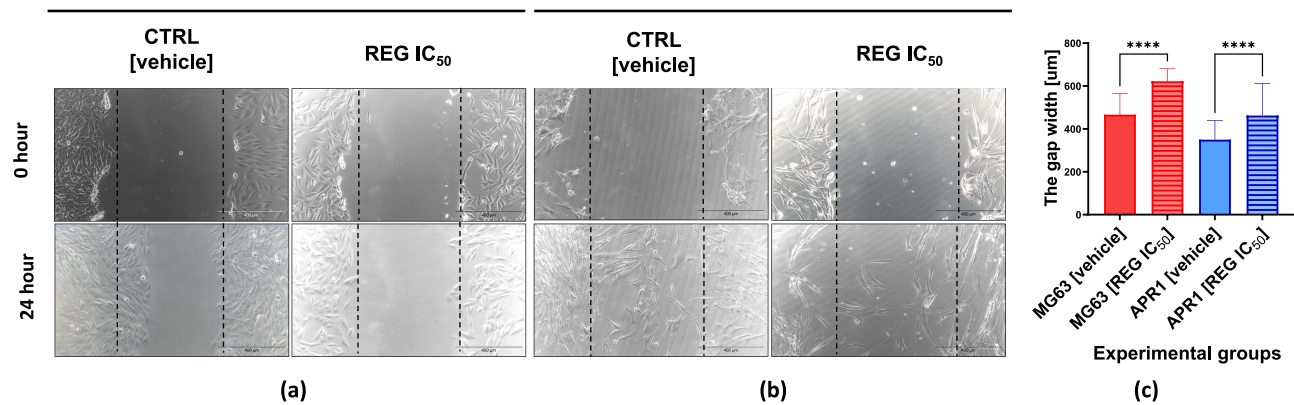


Figure 3 Representative images of the wound healing assay captured for MG63 (a) and for APR1 (b), along with wound gap width measurements (c). The analysis assessed the impact of regorafenib at the IC₅₀ concentration on the invasive and proliferative potential of MG63 and APR1 cell lines measured at starting point (0 hour) and after 24 hours. Images were taken at 100-fold magnification; scale bar: 400 μm. Statistical significance was indicated with asterisks ****p<0.0001. Data are presented as mean ± SD from n = 3 independent biological replicates, with two technical replicates per condition and two images analyzed per each replicate using ImageJ.

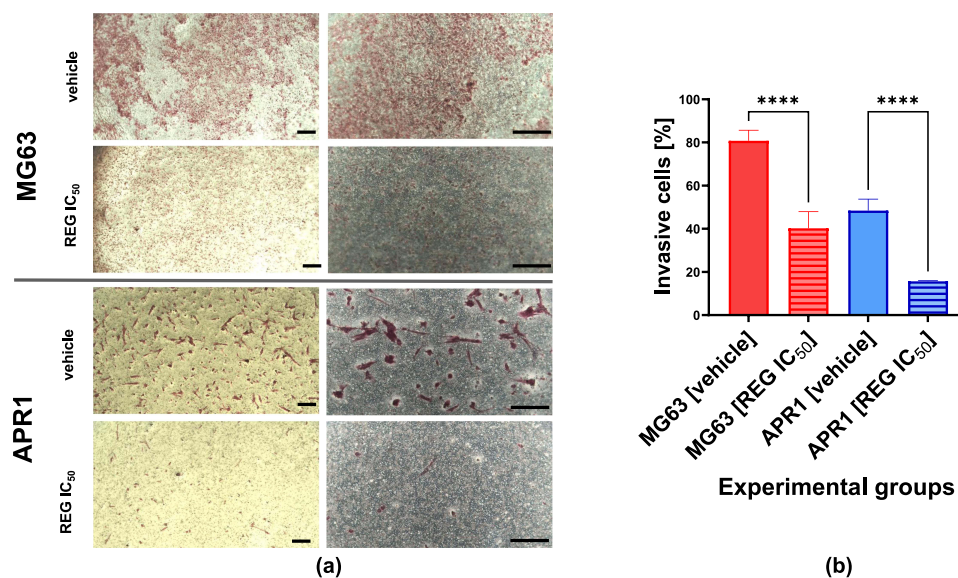


Figure 4 The invasion of the osteosarcoma cell lines MG63 and APR1 is modulated by regorafenib. Representative images (a) and quantitative analysis (b) illustrating the invasive capacity of MG63 and APR1 cells following treatment with regorafenib at the IC₅₀ concentration. Magnification: 40-fold and 100-fold; scale bar: 100 μm. Statistical significance was indicated via asterisks ****p<0.0001. Data are shown as mean ± SD based on three independent biological replicates, each assessed in two technical replicates, with two images per replicate subjected to ImageJ analysis. Results are expressed as the percentage of migrated cells per field and normalized to the corresponding control conditions.

In contrast, the protein expression patterns revealed the opposite trend. Representative Western blot images are shown in panel (g). In MG63 cells, the intracellular levels of BAX and MCL1 were markedly reduced following treatment (Figure 6b and i). Moreover, the BAX/BCL2 ratio, assessed at both the transcript and protein levels (Figure 6e and f), was significantly decreased in MG63 cells, suggesting a shift toward an antiapoptotic balance and a potential survival response despite the transcriptional activation of apoptotic pathways. No significant changes in protein levels were observed in APR1 cells (Figure 6b, d, f, i).

Moreover, to investigate whether regorafenib-induced cytotoxicity is associated with alterations in cell cycle progression, we performed cell cycle analysis with flow cytometry (Figure 7).

In MG63 cells, treatment with regorafenib at the IC₅₀ significantly reduced the proportion of cells in both the G0/G1 and S phases, indicating that their suppressed proliferative activity was associated with drug action. This shift was accompanied by a marked accumulation of cells in the G2/M phase, suggesting that regorafenib induces cell cycle arrest

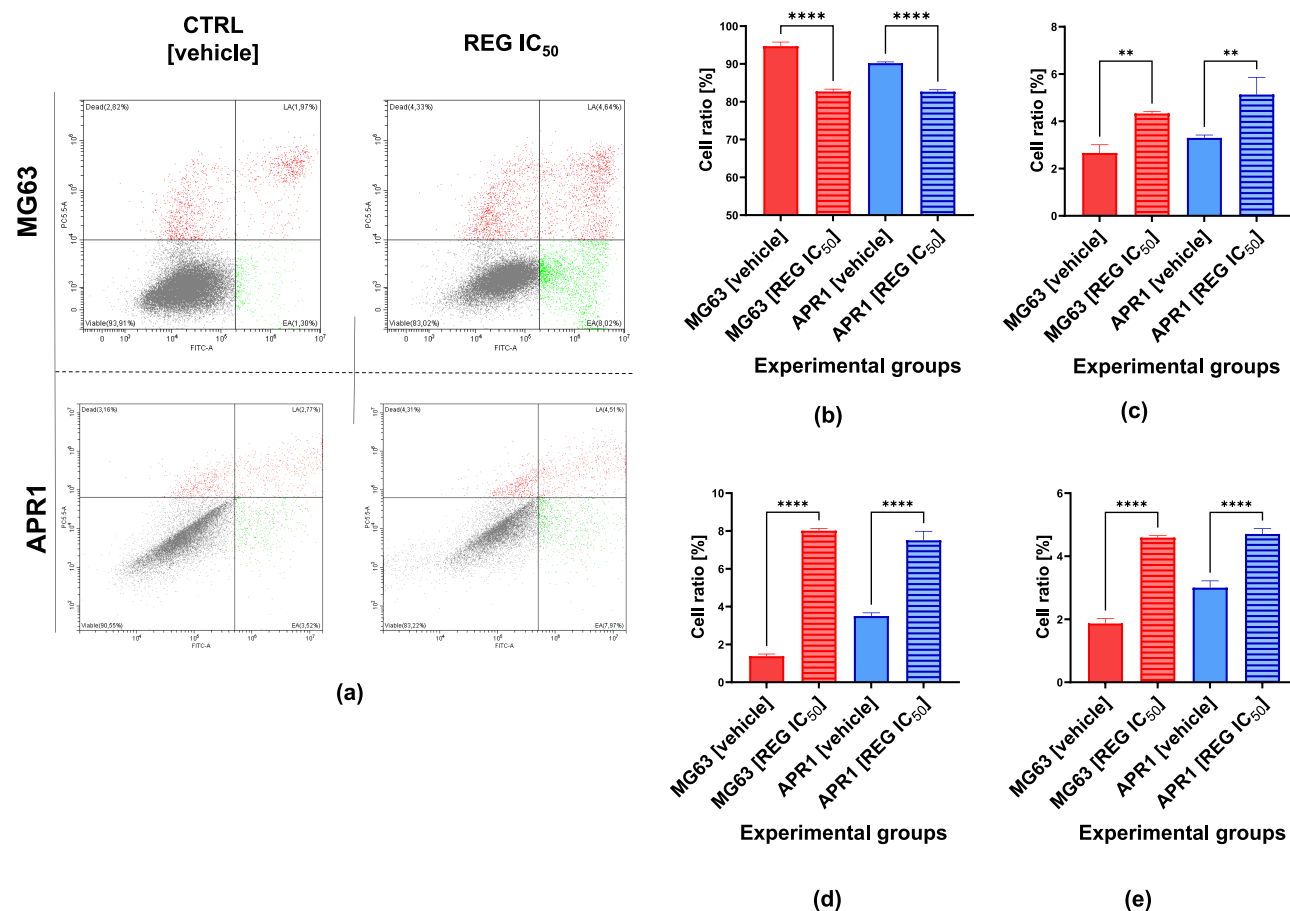


Figure 5 Apoptosis profiles were determined by flow cytometry. Regorafenib at the IC₅₀ decreased the number of viable MG63 and APR1 cells (b) while simultaneously increasing the percentage of necrotic (c), early apoptotic (d), and late apoptotic (e) cells. On the basis of the dot plots (a), four distinct cell populations were identified: viable cells (Annexin V and PI negative), early apoptotic cells [EA] (Annexin V positive, PI negative), late apoptotic cells [LA] (Annexin V and PI positive), and dead (necrotic) cells (Annexin V negative, PI positive). Statistically significant differences are indicated by asterisks (** $p < 0.01$ and **** $p < 0.0001$). Data are presented as mean $\pm$ SD from $n = 2$ biological replicates, each performed in 3 technical replicates.

at this checkpoint (Figure 7 a and b). In contrast, APR1 cells showed no significant changes in the S or G2/M phase following regorafenib treatment. However, a significant increase in the G0/G1 population was observed, which may reflect a protective arrest mechanism (Figure 7a and b).

Moreover, regorafenib treatment led to a marked downregulation of the expression of ncRNAs associated with proliferation and cell cycle progression, including miR-17-5p, miR-21-5p, miR-140-5p, miR-155-5p, and *lncMALAT1* in MG63 cells (Figure 8a–e). This expression profile can be considered as consistent with the observed G2/M arrest and reduced S-phase fraction in these cells, indicating the suppression of proliferative signaling (Figure 7). In contrast, APR1 cells presented upregulation of the same transcripts following regorafenib treatment (Figure 8a–e). Notably, *lncTUG1*, associated with the regulation of cell survival pathways, was upregulated in both cell lines following regorafenib treatment, which may suggest a shared prosurvival response and a mechanism supporting cellular adaptation to treatment-induced stress (Figure 8f).

Regorafenib Interferes with PI3K/AKT/mTOR-Mediated Survival Signaling in Osteosarcoma Cells

The expression of PI3K (phosphatidylinositol 3-kinase), AKT (protein kinase B) and mTOR (mammalian target of rapamycin) was evaluated at the mRNA level (Figure 9b,f,g,k) via RT-qPCR and at the protein level (Figure 9c–e, h–j and l–n) via Western blot analysis. Representative Western blot images are shown in panel (a).

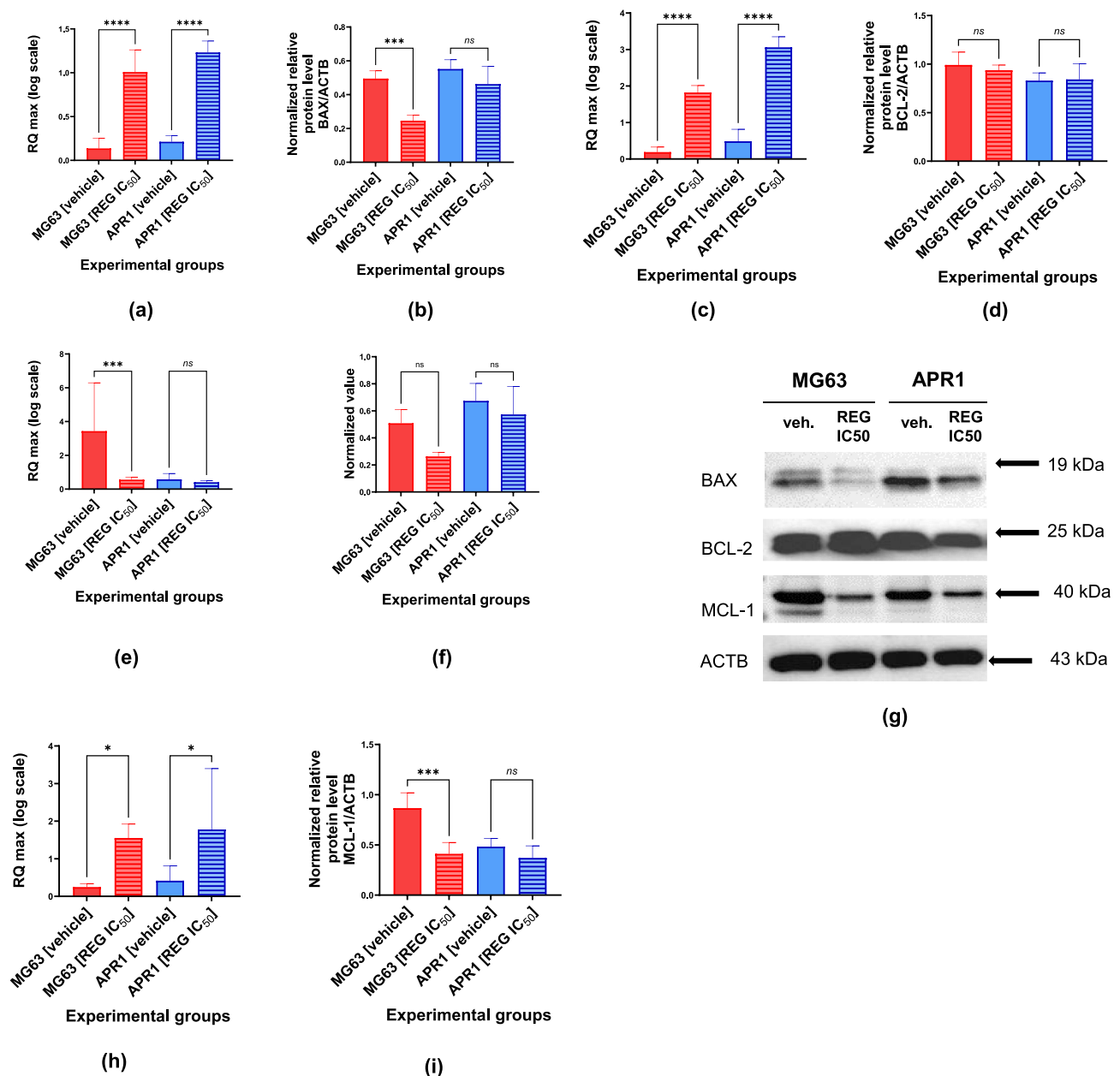


Figure 6 Influence of regorafenib IC₅₀ treatment on the mRNA and protein levels of BAX (a and b), BCL-2 (c and d), and MCL-1 (h and i) in MG63 and APR1 cell lines. Additionally, the BAX/BCL-2 ratio was determined for both mRNA and protein expression (e and f). Gene expression was quantified via RT-qPCR, with normalization to a reference gene, and transcript levels were calculated via the RQmax algorithm. For RT-qPCR experiments, analyses were performed using two independent biological replicates, with each biological sample analyzed in three technical replicates. Representative Western blot images are presented (g), and the corresponding protein levels were quantified through densitometric analysis. Western blot analysis consisted of two independent biological replicates, each performed with two technical replicates. Statistical significance was indicated using asterisks as follows: **p* < 0.05, ****p* < 0.001, *****p* < 0.0001. Differences that were not statistically significant are marked as “ns”.

Regorafenib treatment at the IC₅₀ induced complex, cell line-dependent alterations in the PI3K/AKT/mTOR signaling pathway. While the mRNA levels of mTOR were significantly increased in both MG63 and APR1 cells (Figure 9k), the corresponding protein levels, particularly those of the phosphorylated forms decreased, but significantly only in MG63 cells (Figure 9l–n). Similarly, the phosphorylation of PI3K was decreased significantly in both MG63 and APR1 cells (Figure 9d), although p-PI3K/PI3K ratio was significantly decreased only in MG63 (Figure 9e).

In the examined osteosarcoma cell lines, no uniform pattern of AKT expression was detected in response to regorafenib treatment (Figure 9f–j). In MG63 cells, mRNA expression of both *AKT1* and *AKT2* decreased, whereas in APR1 cells, the expression of both transcripts increased notably (Figure 9f–g). In both cell lines, total AKT protein levels

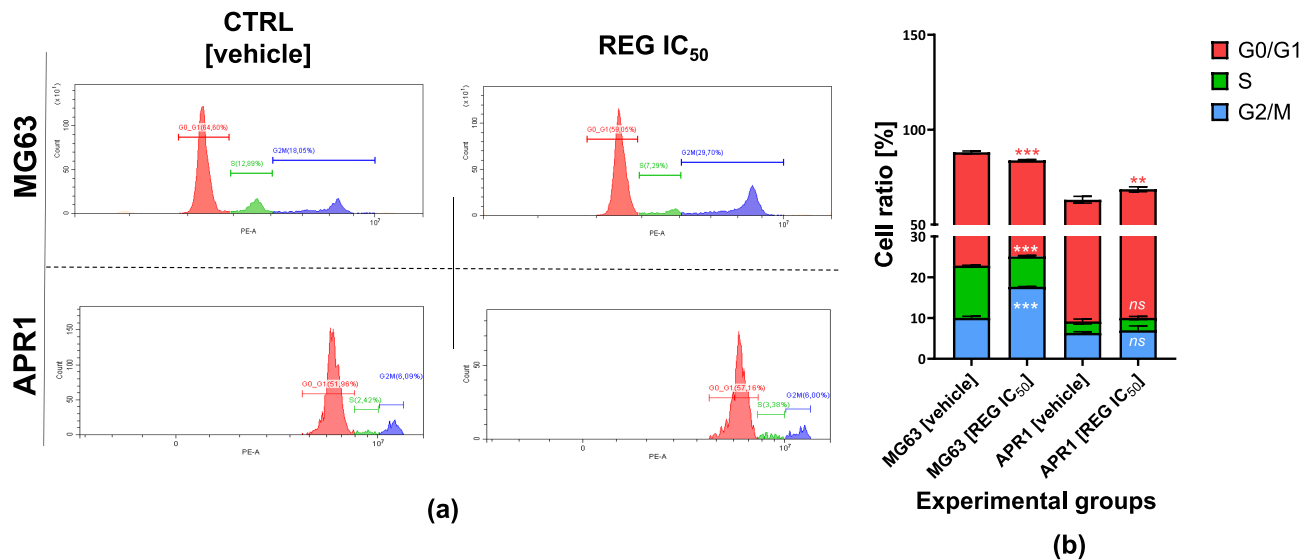


Figure 7 The DNA content distribution in the osteosarcoma cell lines was assessed via flow cytometry. Representative histograms (a) illustrate the proliferative status of cells across the cell cycle phases in the control and regorafenib-treated groups. The cells were classified into three distinct populations: G0/G1 phase, S phase, and G2/M phase (b). Statistically significant differences are indicated by asterisks (** $p < 0.01$ and *** $p < 0.001$), whereas comparisons with no statistically significant differences are marked as “ns”. Values represent the mean $\pm$ SD derived from two independent biological replicates, each including three technical replicates.

were reduced after regorafenib exposure (Figure 9h). A non-significant decrease in phosphorylated AKT (p-AKT) levels was also observed in both models (Figure 9i). The p-AKT/AKT ratio, calculated for MG63 cells, showed a significant increase following regorafenib treatment, indicating a relative enrichment of phosphorylated AKT despite the overall reduction in total AKT protein levels (Figure 9j).

Regorafenib Modulates the Mitochondrial Function and Glycolytic Activity of Osteosarcoma Cells Which Can Be Associated with Their Resistance Phenotype

Given that the PI3K/AKT/mTOR signaling pathway is not only critical for cancer cell survival and proliferation but also plays a key role in the regulation of mitochondrial function and cellular energy metabolism, we sought to determine whether the observed alterations in the pathway activity regulated by regorafenib were associated with functional deficits in ATP production, both in MG63 and APR1 cells.

The bioenergetic profile was assessed by measuring ATP in real time to evaluate the functional impact of regorafenib on cellular energy metabolism (Figure 10). Treatment with regorafenib at its IC₅₀ resulted in a significant reduction in total ATP production in both the MG63 and APR1 cell lines, primarily due to impaired oxidative phosphorylation (OXPHOS; Figure 10a, b, e, f). Notably, in MG63 cells, regorafenib also markedly suppressed glycolytic activity, potentially indicating a dual mechanism of metabolic disruption in those cells (Figure 10c, e). In APR1 cells, regorafenib did not suppress glycolysis-dependent ATP production, which may indicate preserved glycolytic flux during treatment. (Figure 10d and f).

Impact of Regorafenib at the IC₅₀ Concentration on the Expression of Cancer-Associated Genes Assessed By RT² Profiler PCR Arrays

To gain a broader and more integrated understanding of the functional and molecular effects of regorafenib on osteosarcoma cells, we performed a comprehensive gene expression analysis using a targeted panel encompassing genes involved in cancer progression and cellular metabolism (Figure 11). This approach enabled the identification of additional molecular pathways that may underlie the antitumor activity of regorafenib in both osteosarcoma models studied.

In MG63 cells treated with regorafenib at the IC₅₀, ten genes were significantly upregulated compared with those in the control (vehicle) culture. Increased expression was noted for *HMOX1*, *CCND3*, *DSP*, *PINX1*, *DDIT3*, *MAP2K3*,

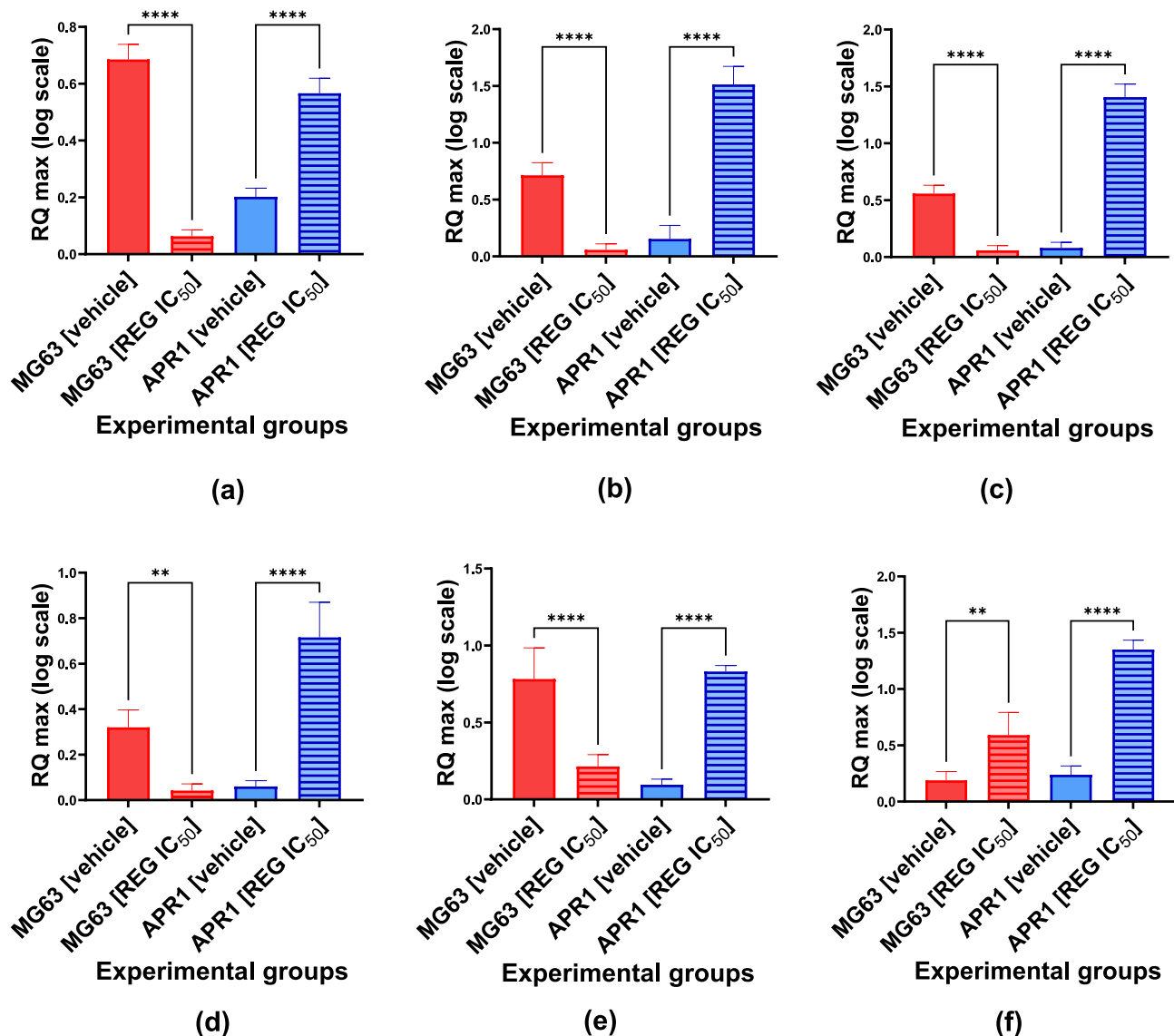


Figure 8 Effect of regorafenib treatment at the IC₅₀ concentration on noncoding RNA expression in MG63 and APR1 cell lines. The expression levels were quantified by RT-qPCR, normalized to a reference gene, and calculated via the RQ Max algorithm. The graphs show the expression of selected microRNAs: miR-17-5p (a), miR-21-5p (b), miR-140-5p (c), miR-155-5p (d) and long noncoding RNAs: *lncMALAT* (e), *lncTUG* (f). Statistically significant differences are indicated by asterisks (**p < 0.01 and ***p < 0.0001). Data represent the mean ± standard deviation of two independent biological experiments, each performed in three technical replicates.

PPP1R15A, *CCND2*, *LPL*, and *EPO*. Conversely, four genes, including *VEGFC*, *CCL2*, *PGF*, and *SERPINF1*, were found to be downregulated in MG63 cells after regorafenib treatment by at least 2-fold (Figure 11a).

In APR1 cells, seven genes, namely, *PPP1R15A*, *PGF*, *CCND2*, *DDIT3*, *HMOX1*, *OCN*, and *GADD45G*, were upregulated more than 2-fold following regorafenib treatment. Concurrently, five genes were downregulated by at least 2-fold: *CDC20*, *MKI67*, *CCL2*, *FOXC2*, and *SERPINF2* (Figure 11b).

Importantly, in both the MG63 and APR1 osteosarcoma cell lines, consistent upregulation of *DDIT3* and *PPP1R15A* (involved in the DNA damage response and repair), *HMOX1* (hypoxia signaling), and *CCND2* (cell cycle regulation) was observed following regorafenib treatment.

In contrast, *CCL2*, a chemokine implicated in angiogenesis, cell migration, and metastatic dissemination, was markedly downregulated in both cell lines (Figure 11a and b). Overall, the data indicate that, in our models and under the tested conditions, regorafenib modulated stress-response markers and signaling pathways linked to metastasis and microenvironmental adaptation.

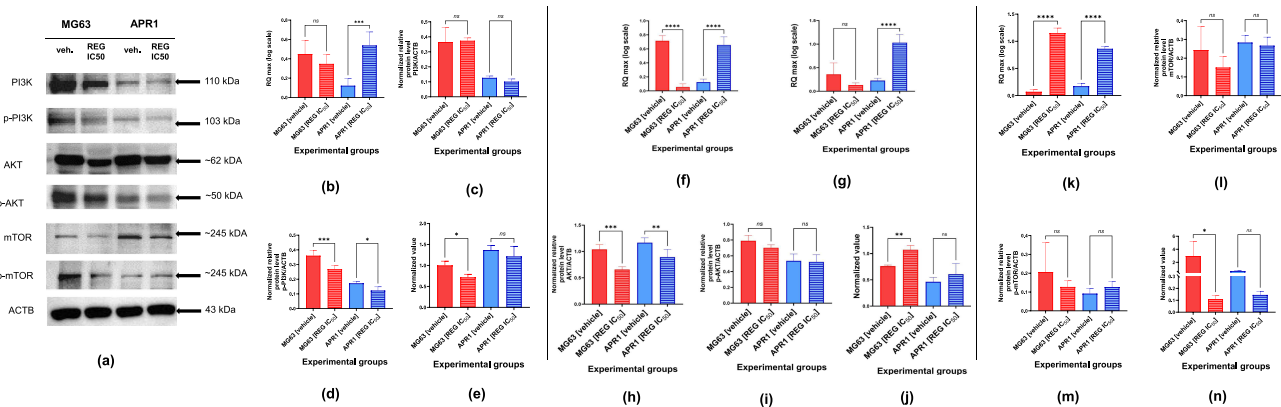


Figure 9 Analysis of the PI3K/AKT/mTOR signaling pathway—the expression of key components of the PI3K/AKT/mTOR pathway was tested at both the mRNA and protein levels. Panel (a) shows representative Western blot images, with corresponding band intensities quantified by densitometric analysis. The expression levels of PI3K, AKT, and mTOR are presented at both the mRNA and protein levels: mRNA expression is shown for PI3K (b), AKT1 (f), AKT2 (g) and mTOR (k); corresponding protein levels are shown for PI3K (c), p-PI3K (d) and p-PI3K/PI3K ratio (e); for AKT (h), p-AKT (i), and p-AKT/AKT ratio (j); for mTOR (l), p-mTOR (m) and p-mTOR/m-TOR ratio (n). Transcript levels were normalized to a reference gene and calculated using the RQ max algorithm. RT-qPCR analyses were conducted on two independent biological replicates, each measured in three technical replicates. Protein expression values were standardized to β -actin. Western blot analyses were performed on two independent biological replicates. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; comparisons without statistical significance are labeled “ns”. Data are expressed as the mean $\pm$ SD of two independent biological experiments.

The expression profiles of all 84 genes involved in cancer-associated signaling pathways were visualized and presented as a heatmap to provide an overview of the global gene expression changes induced by regorafenib treatment (Figure 12).

Discussion

Despite advances in cancer therapy, osteosarcoma continues to pose a persistent clinical challenge, with current treatment strategies frequently insufficient to achieve long-term remission or significantly improve patient outcomes.⁵ However, regorafenib, a multikinase inhibitor, has emerged as a promising candidate for treating osteosarcoma, offering new hope for improved clinical outcomes.^{6–8} Although, ongoing clinical efforts to assess the therapeutic potential of regorafenib in osteosarcoma, the precise molecular and cellular mechanisms driving its antitumor effects remain largely unclear.

In this study, we partially address this gap by evaluating the impact of regorafenib on key cellular parameters of osteosarcoma cells applying models of a well-established MG63 osteosarcoma cell line and a patient-derived primary cell line isolated from bone tumor tissue, ie, APR1, thereby providing a broader view of regorafenib-associated cellular responses across two distinct cellular backgrounds. The literature highlights the importance of including patient-derived models when evaluating the efficacy of new agents against clinically relevant, therapy-experienced tumors, as they better reflect the heterogeneity,²⁹ which is also an approach that supports the rationale behind our experimental design in these preliminary studies.

In our assays, MG63 cells showed lower IC50 values ($26 \pm 3 \mu\text{M}$) than APR1 cells ($42 \pm 12 \mu\text{M}$), consistent with higher sensitivity of MG63 cells under the tested conditions. The relative resistance observed in APR-1 cells may reflect the patient’s prior exposure to chemotherapy, which could have selected for more treatment-adapted tumor cells, as has been previously observed in different types of cancer.^{30,31}

In a study by Pan et al¹⁷ the inhibitory concentration (IC50) of regorafenib for MG63 and U-2 OS cells was reported to range from 5 to 10 μM which differs from concentrations described by us. It is worth noting that this study did not further investigate the effects of regorafenib on MG63 cell viability, nor did it provide mechanistic insights. In turn, in line with our observations, are studies by Bai et al³² reporting an IC50 of 25 μM for regorafenib in HOS-MNNG and MG-63 cell lines determined after 48 h of in vitro exposure. Additionally, Ji et al³³ reported IC50 values exceeding 5 μM for MG63 cells, which illustrates the variability in regorafenib responsiveness reported across studies. Moreover, Sun et al showed that regorafenib reduced viability across a panel of human cancer cell lines, with IC50 values consistently spanning approximately 25–37 μM .³⁴

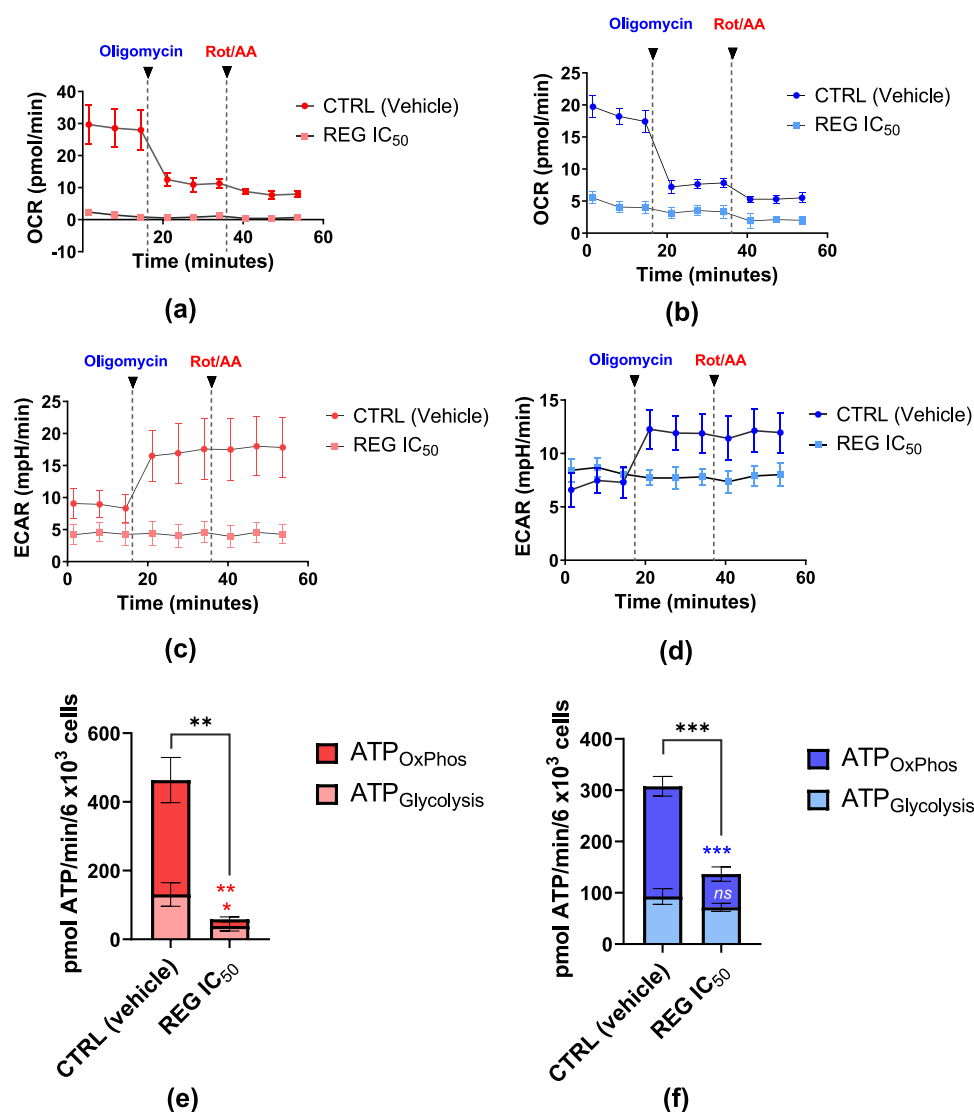


Figure 10 Representative graphs illustrating the oxygen consumption rate (OCR) in the MG63 (a) and APR1 (b) cell lines, as well as the extracellular acidification rate (ECAR) in the MG63 (c) and APR1 (d) cell lines, are presented. Panels (e) and (f) show the calculated rates of ATP production via oxidative phosphorylation (ATP_{OxPhos}) and glycolysis (ATP_{Glycolysis}) in MG63 and APR1 cells, respectively. Statistical significance was denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; differences not reaching statistical significance are labeled "ns". Data are presented as mean $\pm$ SD from the biological replicates. Statistical comparisons between conditions were performed using two-tailed unpaired t-test with a significance threshold of $p < 0.05$.

In the present study, accurate determination of the IC₅₀ values for both the MG63 and APR1 cell lines was further supported by microscopic evaluation of cell growth patterns and confluency. In both the MG63 and APR1 cell lines, regorafenib treatment resulted in an approximately 50% decrease in cell confluency, which aligns with the calculated IC₅₀ values and suggests that the selected concentration appropriately reflects the compound's cytostatic potency.

An important aspect of our study was the evaluation of the impact of regorafenib on the apoptotic profile of osteosarcoma cells. We demonstrated that regorafenib significantly reduces cell viability by inducing both early and late apoptosis in osteosarcoma cells. These findings are partially consistent with those of Pan et al¹⁷ who reported a predominantly late-apoptotic response in U2 OS cells, since our results reveal a broader spectrum of cell death mechanisms. Notably, in addition to apoptosis, we observed a marked increase in necrotic cell populations following regorafenib treatment, suggesting that its cytotoxic activity may involve both programmed and nonprogrammed cell death pathways. Our findings are also in line with reports showing that regorafenib can trigger apoptotic signaling, primarily via PI3K/AKT inhibition and activation of the intrinsic mitochondrial pathway, and in some contexts involving both intrinsic and extrinsic mechanisms.^{34,35}

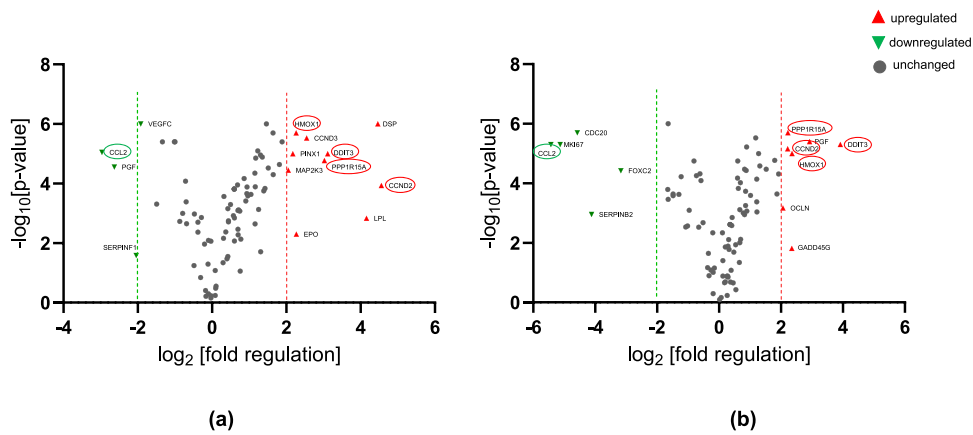


Figure 11 Effect of regorafenib at the IC₅₀ concentration on the expression profile of genes associated with cancer-related pathways in MG63 (a) and APR1 (b) osteosarcoma cell lines. The x-axis of volcano plot represents log₂(fold regulation) and the y-axis -log₁₀(p-value). Upregulated genes are marked with red upward arrows, whereas downregulated genes are marked with green downward arrows. Genes consistently up- or downregulated in both MG-63 and APR-1 cells are additionally highlighted with light-red or light-green circles, respectively. Vertical dashed lines indicate the fold-change cutoff used to define differential expression. Genes showing expression changes of at least 2-fold were classified as differentially expressed. Gene expression levels were normalized to those of reference genes (*ACTB*, *B2M*, *RPLP0*, and *GAPDH*) and calculated using the 2^{-(ΔΔCt)} method. The reactions for this assay were performed using three independent biological replicates, each processed as one technical replicate.

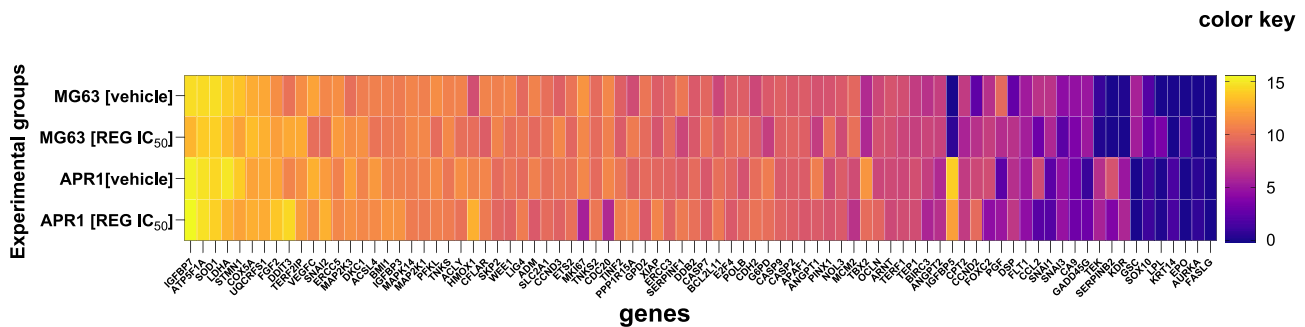


Figure 12 Heatmap illustrating the expression profiles of all 84 genes involved in pathways associated with oncogenesis. The heatmap provides a comprehensive overview of gene expression modulation in MG63 and APR1 osteosarcoma cell lines following treatment with regorafenib at the IC₅₀ concentration.

Pan et al¹⁷ also demonstrated that the proapoptotic effect of regorafenib was associated with caspase-3 activation. In our work, we focused on assessing the effects of regorafenib on the expression of key regulators of apoptosis, namely, BAX, BCL-2, and MCL-1. We demonstrated the downregulation of proapoptotic BAX and MCL-1 protein expression following treatment with regorafenib with simultaneous maintenance of BCL-2 in MG63 cells but not in APR1 cells.

BAX downregulation has been implicated in various stages of tumor development, including initiation, progression, and resistance to therapy. Reduced BAX expression may impair apoptotic signaling, thereby contributing to treatment resistance and limiting the effectiveness of anticancer therapies.³⁶ Notably, a decrease in BAX has been identified as a key mechanism contributing to chemoresistance, where impaired apoptotic signaling can allow malignant cells to evade drug-induced cell death.^{37,38} Importantly, the observed decrease in BAX expression may represent a compensatory response to early apoptotic signaling since under stress conditions, cancer cells may transcriptionally suppress BAX as a survival strategy to evade programmed cell death.³⁹

In turn, MCL-1 is recognized as a critical survival protein in various cell lineages, including cancer cells. Its overexpression has been identified as a key tumor survival mechanism, contributing to protection against chemotherapeutic agents. Suppression of MCL-1 expression via RNA interference (RNAi) has been shown to sensitize cancer cells to chemical treatment.⁴⁰ Consistent with these observations, our data show that regorafenib decreases BAX and MCL-2 intracellular accumulation of proteins but does not significantly alter BCL-2 expression, which may reflect an adaptive cellular mechanism aimed at maintaining survival signaling despite the induction of apoptosis.⁴¹



BCL-2 is a critical antiapoptotic protein, and its upregulation under pathological conditions has been shown to inhibit both apoptosis and autophagy, thereby promoting tumor cell survival and resulting in resistance to treatment.⁴² Obtained by us data may suggest that regorafenib exerts complex effects on the balance between pro- and antiapoptotic agents.

Moreover, the observed changes in apoptotic regulators also highlight an apparent discordance between transcriptional and protein-level regulation, particularly in the case of BAX and MCL-1. While transcriptional profiling suggested activation of stress- and apoptosis-related pathways, regorafenib treatment resulted in reduced protein levels of selected proapoptotic regulators, which was particularly MG63 cells. Such discrepancies between mRNA abundance and protein expression are increasingly recognized as a common feature of stress-adapted cancer cells and may reflect post-transcriptional and post-translational regulatory mechanisms rather than simple transcriptional control.^{43,44}

Importantly, our results revealed that regorafenib has dual anticancer effects, combining proapoptotic and antiproliferative mechanisms. Previously, Pan et al¹⁷ demonstrated that regorafenib may induce a shift in U2O2 cells from the S phase to the G0/G1 phase.

In our study, we thoroughly examined the cell cycle distribution and found that the mechanism of action of regorafenib is more complex. In MG63 osteosarcoma cells, we observed a significant reduction in the proportion of cells in the G0/G1 and S phases, accompanied by a notable accumulation of cells in the G2/M phase following regorafenib treatment. Conversely, in APR1 cells, we noted an increased proportion of cells in the G0/G1 phase, with no significant changes in the S or G2/M phase. A similar dual mechanism of action has been reported for pitavastatin, an antihyperlipidemic drug, whose anticancer activity has been evaluated in cervical cancer cell models. The compound induced sub-G1 and G0/G1-phase arrest in Ca Ski and HeLa cells, both characterized by shorter doubling times, and sub-G1 and G2/M-phase arrest in C-33A cells, reflecting differences in their proliferative profiles.⁴⁵

Moreover, differences in the cell response to regorafenib were also reflected in the different expression patterns of noncoding RNAs (ncRNAs) linked to survival and resistance under metabolic and replicative stress, ie, *lncMALAT1*, miR-17-5p, miR-21-5p, miR-140-5p, and miR-155-5p. These ncRNAs were upregulated in MG63 cells but decreased in APR-1 cells.

A study by Liu et al⁴⁶ highlighted a key role for *lncMALAT1* in mediating chemoresistance in osteosarcoma. These results suggest that doxorubicin treatment induces the upregulation of *MALAT1* and that this upregulation may contribute to the survival and persistence of resistant tumor cells. Importantly, when *MALAT1* was silenced in doxorubicin-resistant U2 OS cells, their sensitivity to chemotherapy was restored, indicating that *MALAT1* functions as a prosurvival regulator and may serve as a therapeutic target to overcome osteosarcoma resistance to therapy.

The role of miR-140-5p in osteosarcoma appears to be context dependent. While high levels of miR-140-5p have been linked to both tumor-suppressive and prosurvival effects depending on the cellular environment, Song et al⁴⁷ demonstrated that miR-140-5p may serve as a biomarker for treatment response, therapeutic efficacy, and prognosis in esophageal squamous cell carcinoma (ESCC). Notably, they reported that its expression was significantly elevated in drug-resistant cells compared with sensitive cells, an observation that aligns with the increased miR-140-5p levels and chemoresistant phenotype of APR1 cells in our study.

In turn, increased levels of miR-17^{48,49}, miR-21-5p,⁵⁰ and miR-155-5p^{51,52} have been linked to enhanced aggressiveness and reduced therapy responsiveness in osteosarcoma.

In our experimental setting, the elevated expression observed in APR1 after regorafenib treatment may therefore reflect intrinsic, background-dependent differences potentially compatible with a more survival-oriented response to regorafenib, involving compensatory pro-survival pathways.

Interestingly, our results also revealed that regorafenib treatment led to upregulation of *lncTUG1* in both the MG63 and APR-1 osteosarcoma cell lines. This finding is particularly noteworthy in light of previous studies showing that *TUG1* functions as an oncogenic lncRNA in osteosarcoma, promoting cell viability, migration, and invasion, at least in part through the upregulation of RUNX2.⁵³ Given the reported pro-oncogenic roles of *TUG1* in several cancer contexts,^{54,55} the obtained results could be compatible with engagement of compensatory programs that support cell survival and/or invasive activity of osteosarcoma.

Given the model-dependent differences observed at the transcript level, and because metabolic state can critically shape drug response, we complemented our molecular and functional analyses with bioenergetic profiling to provide an additional functional layer for interpreting regorafenib-associated effects across both models.

In MG63 cells, regorafenib treatment markedly inhibited ATP production via both oxidative phosphorylation (OxPhos) and glycolysis, indicating a profound disruption of energy metabolism. In contrast, ATP synthesis in APR1 cells is preserved primarily via glycolysis. Previously, regorafenib was shown to uncouple oxidative phosphorylation, reduce ATP levels, and disrupt the mitochondrial membrane potential in isolated rat liver mitochondria and primary hepatocytes. These mitochondrial effects trigger mitochondrial permeability transition (MPT) and necrosis, while adaptive responses such as AMPK activation and autophagy are mobilized as prosurvival mechanisms.⁵⁶ This study underscores the compound's capacity to interfere with energy metabolism in nontumor cell models; however, such information remains limited in the context of cancer cells. Even so, it is well established that therapy-resistant cancer cells often shift toward glycolytic energy production over oxidative phosphorylation, which can support survival and proliferation when mitochondrial function is compromised, a framework that may align with the response of the patient-derived APR1 model in our experimental setting.⁵⁷

In addition to the observed bioenergetic, further variations in the response of MG63 and APR1 cells to regorafenib were detected in the activity of the PI3K/AKT/mTOR signaling pathway, a key pathway involved in the regulation of proliferation, survival, and therapy resistance.⁵⁸

Constitutive activation of the PI3K/AKT/mTOR pathway is commonly reported in osteosarcoma and is associated with increased proliferation and metastasis and reduced sensitivity to treatment.⁴⁰ In our study, we demonstrated that regorafenib treatment resulted in a reduction in the phosphorylation levels of the PI3K and mTOR proteins, particularly in the MG63 cell line. In contrast, APR1 cells showed no statistically significant changes in PI3K/AKT/mTOR phosphorylation following regorafenib exposure evidenced by reference to the total protein detected (ratio). However, a significant decrease in total AKT (pan-AKT1/2/3) was observed in both cell lines. This finding suggests a possible decoupling of mTOR activity from upstream AKT signaling and potential activation of alternative compensatory pathways.⁵⁹ Thus, to identify potential molecular targets and signaling pathways involved in the cellular response to regorafenib, we performed high-throughput screening of 84 cancer-related genes via a pathway-focused qPCR array.

The analysis of both cell lines revealed the activation of genes preserving cellular homeostasis under drug pressure, such as *DDIT3*, *PPP1R15A*, *HMOX1*, and *CCND2*.

The upregulation of *DDIT3* observed in both MG63 and APR-1 cells following regorafenib treatment may suggest activation of the ER stress response pathway since *DDIT3* (*CHOP*) is a canonical marker of sustained ER stress and is commonly linked to growth arrest and pro-apoptotic signaling when adaptive UPR mechanisms are insufficient.⁶⁰ In addition to its canonical role in promoting apoptosis under unresolved stress, *DDIT3* has been also shown to modulate cell cycle dynamics, partly through its interaction with *CDK2* and interference with cyclin-CDK complex formation. Its sustained expression appears to be essential for the continued expansion of cancer stem cells by supporting proper cell cycle progression and proliferation while minimizing the accumulation of DNA damage.⁶¹

In our study, we also observed an upregulation of *HMOX1* in both MG63 and APR-1 cells following regorafenib treatment. Given that *HMOX1* is a well-established stress-responsive gene and a key component of cellular antioxidant defence (including limiting the accumulation of reactive oxygen species, ROS),⁶² this increase may indicate activation of adaptive cytoprotective mechanisms under the applied conditions. Such a mechanism has been observed, among others, in mantle cell lymphoma cells, where silencing *BACH2*, a transcription factor involved in regulating oxidative stress-responsive genes, led to increased *HMOX1* expression and was reported to facilitate bortezomib resistance, inter alia by inducing cytoprotective autophagy and maintaining ROS at low levels. Beyond its antioxidant properties, *HMOX1* also modulates key processes, such as cell proliferation, inflammatory signaling, angiogenesis, and ferroptosis, which are particularly relevant in the tumor microenvironment.^{63,64} Thus, increased *HMOX1* after regorafenib exposure may reflect engagement of a broader stress-adaptive programme. For instance, we also noted a concurrent induction of additional stress-response transcripts. In addition, the upregulation of *PPP1R15A* (*GADD34*). *PPP1R15A*, a regulatory subunit of protein phosphatase 1, is a well-established downstream target of the integrated stress response and has been shown to restore protein synthesis under ER and metabolic stress.⁶⁵ Notably, recent findings in gastric cancer demonstrated that

PPP1R15A is transcriptionally activated by the transcription factor JUN under glucose deprivation, promoting autophagy and supporting cell survival under nutrient-poor conditions.⁶⁶

Interestingly, regorafenib treatment also led to significant downregulation of *CCL2* expression in both the MG63 and APR-1 osteosarcoma cell lines. *CCL2* (C–C motif chemokine ligand 2) is widely recognized for its role in promoting tumor invasion and metastasis across multiple cancer types, including osteosarcoma. It has been shown to be abundantly secreted by both murine and human OS cells with high metastatic potential and is particularly enriched in extracellular vesicles (EVs) derived from these aggressive cell populations. Through paracrine signaling and EV-mediated communication, *CCL2* contributes to remodeling of the tumor microenvironment, recruitment of protumorigenic immune cells, and facilitation of metastatic dissemination.⁶⁷ In light of this, the reduced *CCL2* expression would therefore be consistent with the attenuated migratory and invasive phenotype observed after regorafenib exposure.

Indeed, regorafenib has previously been characterized for its inhibitory effects on the migration and invasion of various cancer cell lines, including hepatocellular carcinoma,⁶⁸ melanoma,⁶⁹ and human bladder carcinoma cells.⁷⁰ Zhang et al demonstrated that regorafenib significantly reduced the metastatic potential of Huh7 and PLC hepatocellular carcinoma cells in a concentration-dependent manner.⁶⁸ Similarly, Xuan et al reported that regorafenib significantly decreased the number of migrating melanoma cells and impaired wound closure in cultures of Sk-Mel-2 and Sk-Mel-28 cell lines.⁶⁹ In addition, Hsu et al reported that regorafenib significantly reduced both the migratory and invasive capacities of TSGH 8301 bladder carcinoma cells.⁷⁰ Importantly, the antimetastatic potential of regorafenib is not limited to in vitro observations; clinical evidence also supports its efficacy in patients with relapsed metastatic osteosarcoma.¹⁶

Taken together, our findings indicate that regorafenib exerts its antitumor activity through a combination of proapoptotic, antiproliferative, and antimetastatic mechanisms, which may be mediated by disrupted mitochondrial metabolism and ATP production, suppression of survival signaling, and transcriptional reprogramming under stress. The divergent responses between MG63 and APR1 cells highlight the potential role of cell heterogeneity in shaping differential sensitivity to regorafenib.

Despite the differences in cellular responses to the drug, our data reveal several shared molecular effects of regorafenib, including the upregulation of stress-response genes (*DDIT3*, *PPP1R15A*, *HMOX1*, *CCND2*) and the downregulation of the prometastatic chemokine *CCL2*, suggesting that key elements of the drug's mechanism of action are conserved across cell lines. Moreover, distinct patterns of noncoding RNA expression and differential modulation of the PI3K/AKT/mTOR signaling pathway point to the presence of cell-specific adaptive programs that shape the drug response in a context-dependent manner.

Conclusion

Our study provides a comprehensive, but preliminary evaluation of the cellular and molecular mechanisms underlying the antitumor activity of regorafenib in osteosarcoma using both established (MG63) and patient-derived (APR-1) cell models. Our findings reveal that regorafenib has a potent antiproliferative, proapoptotic, and antimetastatic effect toward osteosarcoma cells. Its antitumor activity may be linked to the induction of apoptosis, disruption of mitochondrial bioenergetics, induction of cell-cycle arrest, modulation of the PI3K/AKT/mTOR signalling pathway, and changes in both coding and non-coding gene expression profiles associated with cell survival and stress-adaptive responses in osteosarcoma cells.

While established cell line MG63, displayed increased sensitivity to regorafenib, APR1 cells presented features of resistance, including preserved glycolytic ATP production and upregulation of survival-associated noncoding RNAs, which may be related to the greater biological heterogeneity typically observed in clinically derived models.

Our study is preliminary and hypothesis-generating, as it is based on controlled in vitro conditions and a limited set of osteosarcoma models, which cannot fully recapitulate the heterogeneity and complexity of patient tumors. Consequently, while the IC50 values established here provide a useful in vitro reference point for comparing relative sensitivity and response under the defined assay conditions, they cannot be straightforwardly extrapolated to clinical dosing, efficacy; Further validation across broader panels of patient-derived models and, ultimately, in vivo/clinical datasets will be necessary to establish translational relevance. Nevertheless, our study helps to narrow this knowledge gap by identifying both shared and divergent molecular pathways associated with osteosarcoma cell responses to regorafenib and may highlight candidate signatures for further validation as potential markers of drug response.

Abbreviations

ACTB, beta-actin; AKT, protein kinase B; ATP, adenosine triphosphate; B2M, beta-2-microglobulin; BAX, BCL2 associated X apoptosis regulator; BCA, bicinchoninic acid assay kit; BCL2, BCL2 apoptosis regulator; BRAF, b-raf proto-oncogene; BSA, bovine serum albumin; CCL2, C-C motif chemokine ligand 2; CCND2, cyclin D2; CCND3, cyclin D3; CDC20 - cell division cycle 20; CGM, complete growth medium; DDIT3, DNA damage inducible transcript 3; DMEM LG - Dulbecco's Modified Eagle Medium Low Glucose; DMSO, dimethyl sulfoxide; DSP, desmoplakin; ECAR, extracellular acidification rate; EMEM, Minimum Essential Medium Eagle; EPO, erythropoietin; Evs, extracellular vesicles; FAS, apoptosis antigen 1; FASL, apoptosis antigen 1 ligand; FBS, fetal bovine serum; FGFR1/2, fibroblast growth factor receptor 1/2; FOXC2, forkhead box protein C2; GADD45G, growth arrest and DNA damage inducible gamma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMOX1, heme oxygenase 1; KIT, proto-oncogene, receptor tyrosine kinase; lncMALAT1, metastasis-associated in lung adenocarcinoma transcript 1; lncTUG, taurine upregulated 1; LPL, lipoprotein lipase; MAP2K3, mitogen-activated protein kinase kinase 3; MAPK, mitogen-activated protein kinase; MCL-1, MCL1 apoptosis regulator, BCL2 family member; MKI67, marker of proliferation Ki-67; MMP-9, matrix metalloproteinase 9; MPT, mitochondrial permeability transition; mTOR, mammalian target of rapamycin; NCCN, national comprehensive cancer network; ncRNA, noncoding RNA; OCLN, occludin; OXPHOS, oxidative phosphorylation, P/S, penicillin-streptomycin; PDGFR, α/β - platelet-derived growth factor receptor α/β ; PFA, paraformaldehyde; PGF, placental growth factor; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PINX1, PIN2/TERF1-interacting telomerase inhibitor 1; PPP1R15A, GADD34 or protein phosphatase 1 regulatory subunit 15A; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; RAF-1; Raf-1 proto-oncogene; RET, ret proto-oncogene; RPLP0, ribosomal protein, large, P0; RTKs, tyrosine kinase receptors; RUNX2, RUNX family transcription factor 2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SERPINB2 - plasminogen activator inhibitor 2; SERPINF1 - serpin peptidase inhibitor; TBST, tris-buffered saline with Tween 20, TKIs, tyrosine kinase inhibitors; TME, tumor micro-environment; VEGF, vascular endothelial growth factor; VEGFC, vascular endothelial growth factor C; VEGFR1-3, vascular endothelial growth factor receptor 1-3; XIAP, X-linked inhibitor of apoptosis.

Data Sharing Statement

All the data generated or analyzed during this study are included in this published article or are available from the corresponding author upon reasonable request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests in this work.



References

- Maleki M, Golchin A, Alemi F, et al. Cytotoxicity and apoptosis of nanoparticles on osteosarcoma cells using doxorubicin and methotrexate: a systematic review. *Eur J Pharmacol.* 2021;904(174131):174131. doi:10.1016/j.ejphar.2021.174131
- Zhang B, Zhang Y, Li R, Li J, Lu X, Zhang Y. The efficacy and safety comparison of first-line chemotherapeutic agents (high-dose methotrexate, doxorubicin, cisplatin, and ifosfamide) for osteosarcoma: a network meta-analysis. *J Orthop Surg Res.* 2020;15(1):51. doi:10.1186/s13018-020-1576-0
- Marina NM, Smeland S, Bielack SS, et al. Comparison of MAPIE versus MAP in patients with a poor response to preoperative chemotherapy for newly diagnosed high-grade osteosarcoma (EURAMOS-1): an open-label, international, randomised controlled trial. *Lancet Oncol.* 2016;17(10):1396–1408. doi:10.1016/S1470-2045(16)30214-5
- Czarnecka AM, Synoradzki K, Firliej W, et al. Molecular biology of osteosarcoma. *Cancers.* 2020;12(8):2130. doi:10.3390/cancers12082130
- Shoaib Z, Fan TM, Irudayaraj JMK. Osteosarcoma mechanobiology and therapeutic targets. *Br J Pharmacol.* 2022;179(2):201–217. doi:10.1111/bph.15713
- Han Y, Xie J, Wang Y, Liang X, Xie Y. Efficacy and safety of regorafenib in the treatment of bone sarcomas: systematic review and meta-analysis. *BMC Cancer.* 2025;25(1):302. doi:10.1186/s12885-025-13722-y
- Assi A, Farhat M, Hachem MCR, et al. Tyrosine kinase inhibitors in osteosarcoma: adapting treatment strategies. *J Bone Oncol.* 2023;43(100511):100511. doi:10.1016/j.jbo.2023.100511
- Wang H, Jin X, Gao Y, et al. Reprogramming tumor microenvironment via dual targeting co-delivery of regorafenib and alpha-difluoromethylornithine in osteosarcoma. *Cancer Nanotechnol.* 2023;14(1). doi:10.1186/s12645-023-00186-7
- Subramonian D, Phanhtilath N, Rinehardt H, et al. Regorafenib is effective against neuroblastoma in vitro and in vivo and inhibits the RAS/MAPK, PI3K/Akt/mTOR and Fos/Jun pathways. *Br J Cancer.* 2020;123(4):568–579. doi:10.1038/s41416-020-0905-8
- Yu CC, Huang SY, Chang SF, Liao KF, Chiu SC. The synergistic anti-cancer effects of NVP-BE235 and regorafenib in hepatocellular carcinoma. *Molecules.* 2020;25(10):2454. doi:10.3390/molecules25102454
- Hsu FT, Chiang IT, Wang WS. Induction of apoptosis through extrinsic/intrinsic pathways and suppression of ERK/NF- κ B signalling participate in anti-glioblastoma of imipramine. *J Cell Mol Med.* 2020;24(7):3982–4000. doi:10.1111/jcmm.15022
- Van Cutsem E, Martinelli E, Cascinu S, et al. Regorafenib for patients with metastatic colorectal cancer who progressed after standard therapy: results of the large, single-arm, open-label phase IIIb CONSIGN study. *Oncologist.* 2019;24(2):185–192. doi:10.1634/theoncologist.2018-0072
- Fondevila F, Méndez-Blanco C, Fernández-Palanca P, González-Gallego J, Mauriz JL. Anti-tumoral activity of single and combined regorafenib treatments in preclinical models of liver and gastrointestinal cancers. *Exp Mol Med.* 2019;51(9):1–15. doi:10.1038/s12276-019-0308-1
- Harris MA, Hawkins CJ. Recent and ongoing research into metastatic osteosarcoma treatments. *Int J Mol Sci.* 2022;23(7):3817. doi:10.3390/ijms23073817
- Duffaud F, Mir O, Boudou-Rouquette P, et al. Efficacy and safety of regorafenib in adult patients with metastatic osteosarcoma: a non-comparative, randomised, double-blind, placebo-controlled, Phase 2 study. *Lancet Oncol.* 2019;20(1):120–133. doi:10.1016/S1470-2045(18)30742-3
- Davis LE, Bolejack V, Ryan CW, et al. Randomized double-blind phase II study of regorafenib in patients with metastatic osteosarcoma. *J Clin Oncol.* 2019;37(16):1424–1431. doi:10.1200/JCO.18.02374
- Pan PJ, Liu YC, Hsu FT. Protein kinase B and extracellular signal-regulated kinase inactivation is associated with regorafenib-induced inhibition of osteosarcoma progression in vitro and in vivo. *J Clin Med.* 2019;8(6):900. doi:10.3390/jcm8060900
- Chow T, Humble W, Lucarelli E, et al. Feasibility and barriers to rapid establishment of patient-derived primary osteosarcoma cell lines in clinical management. *iScience.* 2024;27(9):110251. doi:10.1016/j.isci.2024.110251
- Tippett VL, Tattersall L, Ab Latif NB, Shah KM, Lawson MA, Gartland A. The strategy and clinical relevance of in vitro models of MAP resistance in osteosarcoma: a systematic review. *Oncogene.* 2023;42(4):259–277. doi:10.1038/s41388-022-02529-x
- Zhang J, Yin J, Liu L, et al. Metabolic reprogramming in osteosarcoma: recent advances and therapeutic opportunities. *Front Oncol.* 2022;12:857686. doi:10.3389/fonc.2022.857686
- Viale A, Corti D, Draetta GF. The Warburg effect: evolving interpretations of an established concept. *Cell Metab.* 2022;34(8):1236–1250. doi:10.1016/j.cmet.2021.11.019
- Śmieszek A, Marcinkowska K, Małas Z, et al. Identification and characterization of stromal-like cells with CD207+/low CD1a+/low phenotype derived from histiocytic lesions—a perspective in vitro model for drug testing. *BMC Cancer.* 2024;24(1). doi:10.1186/s12885-023-11807-0
- Śmieszek A, Marcinkowska K, Pielok A, et al. Obesity affects the proliferative potential of equine endometrial progenitor cells and modulates their molecular phenotype associated with mitochondrial metabolism. *Cells.* 2022;11(9):1437. doi:10.3390/cells11091437
- Zhou X, Li Y, Zhang Q, et al. miR-451a promotes cell growth, migration and EMT in osteosarcoma by regulating YTHDC1-mediated m6A methylation and activating AKT/mTOR signalling. *J Bone Oncol.* 2022;100507. doi:10.1016/j.jbo.2022.100507
- Wang X, Decker CC, Zechner L, et al. In vitro wound healing of tumor cells: inhibition of cell migration by selected cytotoxic alkaloids. *BMC Pharmacol Toxicol.* 2019;20(1):4. doi:10.1186/s40360-018-0284-4
- Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9(7):676–682. doi:10.1038/nmeth.2019
- Ye J, Coulouris G, Zaretskaya I, et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinf.* 2012;13(1):134. doi:10.1186/1471-2105-13-134
- Stein C. A two-sample test for a linear hypothesis whose power is independent of the variance. *Ann Math Stat.* 1945;16(3):243–258. doi:10.1214/aoms/117731088
- Genta S, Coburn B, Cescon DW, Spreafico A. Patient-derived cancer models: valuable platforms for anticancer drug testing. *Front Oncol.* 2022;12:976065. doi:10.3389/fonc.2022.976065
- Qin S, Jiang J, Lu Y, et al. Emerging role of tumor cell plasticity in modifying therapeutic response. *Signal Transduct Target Ther.* 2020;5(1):228. doi:10.1038/s41392-020-00313-5
- Di Nicolantonio F, Mercer SJ, Knight LA, et al. Cancer cell adaptation to chemotherapy. *BMC Cancer.* 2005;5(1):78. doi:10.1186/1471-2407-5-78
- Bai G, Zhao S, Zhao M, Shen L, Chen W. The phosphatase CTDSPL2 promotes proliferation, invasion, metastasis and regorafenib resistance in osteosarcoma. *J Bone Oncol.* 2025;52(100684):100684. doi:10.1016/j.jbo.2025.100684

33. Ji Y, Harris MA, Newton LM, et al. Osteosarcoma cells depend on MCL-1 for survival, and osteosarcoma metastases respond to MCL-1 antagonism plus regorafenib in vivo. *BMC Cancer*. 2024;24(1):1350. doi:10.1186/s12885-024-13088-7
34. Sun B, Chen H, Wang X, Chen T. Regorafenib induces Bim-mediated intrinsic apoptosis by blocking AKT-mediated FOXO3a nuclear export. *Cell Death Discov*. 2023;9(1):37. doi:10.1038/s41420-023-01338-9
35. Chiang CH, Chung JG, Hsu FT. Regorafenib induces extrinsic/intrinsic apoptosis and inhibits MAPK/NF- κ B-modulated tumor progression in bladder cancer in vitro and in vivo. *Environ Toxicol*. 2019;34(6):679–688. doi:10.1002/tox.22734
36. Eischen CM, Rehg JE, Korsmeyer SJ, Cleveland JL. Loss of Bax alters tumor spectrum and tumor numbers in ARF-deficient mice. *Cancer Res*. 2002;62(7):2184–2191.
37. Zhang Z, Hou L, Liu D, Luan S, Huang M, Zhao L. Directly targeting BAX for drug discovery: therapeutic opportunities and challenges. *Acta Pharm Sin B*. 2024;14(6):2378–2401. doi:10.1016/j.apsb.2024.02.010
38. Perego P, Giarola M, Righetti SC, et al. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res*. 1996;56(3):556–562.
39. Chandrika BB, Maney SK, Lekshmi SU, et al. Bax deficiency mediated drug resistance can be reversed by endoplasmic reticulum stress induced death signaling. *Biochem Pharmacol*. 2010;79(11):1589–1599. doi:10.1016/j.bcp.2010.01.032
40. Peddaboina C, Jupiter D, Fletcher S, et al. The downregulation of Mcl-1 via USP9X inhibition sensitizes solid tumors to Bcl-xl inhibition. *BMC Cancer*. 2012;12(1):541. doi:10.1186/1471-2407-12-541
41. Ierano C, Chakraborty AR, Nicolae A, et al. Loss of the proteins Bak and Bax prevents apoptosis mediated by histone deacetylase inhibitors. *Cell Cycle*. 2013;12(17):2829–2838. doi:10.4161/cc.25914
42. Palabiyik AA. The role of Bcl 2 in controlling the transition between autophagy and apoptosis (Review). *Mol Med Rep*. 2025;32(1):1–9. doi:10.3892/mmr.2025.13537
43. Wang Q, Lin J. Homeostasis of mRNA concentrations through coupling transcription, export, and degradation. *iScience*. 2024;27(8):110531. doi:10.1016/j.isci.2024.110531
44. Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. *Cell*. 2016;165(3):535–550. doi:10.1016/j.cell.2016.03.014
45. Chen YH, Wu JX, Yang SF, Wu YC, Hsiao YH. Molecular mechanisms underlying the anticancer properties of pitavastatin against cervical cancer cells. *Int J Mol Sci*. 2024;25(14):7915. doi:10.3390/ijms25147915
46. Liu C, Han X, Li B, et al. MALAT-1 is associated with the doxorubicin resistance in U-2OS osteosarcoma cells. *Cancer Manag Res*. 2021;13:6879–6889. doi:10.2147/CMAR.S304922
47. Song B, Liu X, Dong H, Roy R. MiR-140-3P induces chemotherapy resistance in esophageal carcinoma by targeting the NFYA-MDR1 axis. *Appl Biochem Biotechnol*. 2023;195(2):973–991. doi:10.1007/s12010-022-04139-5
48. Gao Y, Luo LH, Li S, Yang C. miR-17 inhibitor suppressed osteosarcoma tumor growth and metastasis via increasing PTEN expression. *Biochem Biophys Res Commun*. 2014;444(2):230–234. doi:10.1016/j.bbrc.2014.01.061
49. Wu D, Zhang H, Ji F, Ding W. MicroRNA-17 promotes osteosarcoma cells proliferation and migration and inhibits apoptosis by regulating SASH1 expression. *Pathol Res Pract*. 2019;215(1):115–120. doi:10.1016/j.prp.2018.10.012
50. Zhou L, Lu Y, Liu JS, et al. The role of miR-21/RECK in the inhibition of osteosarcoma by curcumin. *Mol Cell Probes*. 2020;51(101534):101534. doi:10.1016/j.mcp.2020.101534
51. Chen L, Jiang K, Jiang H, Wei P. miR-155 mediates drug resistance in osteosarcoma cells via inducing autophagy. *Exp Ther Med*. 2014;8(2):527–532. doi:10.3892/etm.2014.1752
52. Lu S, Liao QS, Tang L. MiR-155 affects osteosarcoma cell proliferation and invasion through regulating NF- κ B signaling pathway. *Eur Rev Med Pharmacol Sci*. 2018;22(22):7633–7639. doi:10.26355/eurrev_201811_16380
53. Sheng K, Li Y. LncRNA TUG1 promotes the development of osteosarcoma through RUNX2. *Exp Ther Med*. 2019;18(4):3002–3008. doi:10.3892/etm.2019.7880
54. An X, Wu W, Wang P, et al. Long noncoding RNA TUG1 promotes malignant progression of osteosarcoma by enhancing ZBTB7C expression. *Biomed J*. 2024;47(3):100651. doi:10.1016/j.bj.2023.100651
55. Li Z, Shen J, Chan MT, Wu WK. TUG1: a pivotal oncogenic long non-coding RNA of human cancers. *Cell Prolif*. 2016;49(4):471–475. doi:10.1111/cpr.12269
56. Weng Z, Luo Y, Yang X, et al. Regorafenib impairs mitochondrial functions, activates AMP-activated protein kinase, induces autophagy, and causes rat hepatocyte necrosis. *Toxicology*. 2015;327:10–21. doi:10.1016/j.tox.2014.11.002
57. Yadav N, Kumar S, Marlowe T, et al. Oxidative phosphorylation-dependent regulation of cancer cell apoptosis in response to anticancer agents. *Cell Death Dis*. 2015;6(11):e1969. doi:10.1038/cddis.2015.305
58. Glaviano A, Foo ASC, Lam HY, et al. PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Mol Cancer*. 2023;22(1):138. doi:10.1186/s12943-023-01827-6
59. Memmott RM, Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. *Cell Signal*. 2009;21(5):656–664. doi:10.1016/j.cellsig.2009.01.004
60. Zhu SL, Qi M, Chen MT, et al. A novel DDIT3 activator dehydroevodiamine effectively inhibits tumor growth and tumor cell stemness in pancreatic cancer. *Phytomedicine*. 2024;128:155377. doi:10.1016/j.phymed.2024.155377
61. Park SY, Lee CJ, Choi JH, et al. The JAK2/STAT3/CCND2 Axis promotes colorectal Cancer stem cell persistence and radioresistance. *J Exp Clin Cancer Res*. 2019;38(1):399. doi:10.1186/s13046-019-1405-7
62. Chudy P, Kochan J, Wawro M, et al. Heme oxygenase-1 protects cells from replication stress. *Redox Biol*. 2024;75:103247. doi:10.1016/j.redox.2024.103247
63. Lin H, Chen X, Zhang C, et al. EF24 induces ferroptosis in osteosarcoma cells through HMOX1. *Biomed Pharmacother*. 2021;136(111202):111202. doi:10.1016/j.biopha.2020.111202
64. Kharb S, Halder M, Kundu ZS. Heme oxygenase-1 in osteosarcoma. *J Cancer Res Ther*. 2020;16(4):874–877. doi:10.4103/jcr.JCRT_419_17
65. Wang R, Zhang Y, Guo S, et al. Single-cell RNA sequencing reveals the suppressive effect of PPP1R15A inhibitor Sephin1 in antitumor immunity. *iScience*. 2023;26(2):105954. doi:10.1016/j.isci.2023.105954

66. Cui Y, Cao X, Zhang Y, et al. Protein phosphatase 1 regulatory subunit 15 A (PPP1R15A) promoted the progression of gastric cancer by activating cell autophagy under energy stress. *J Exp Clin Cancer Res*. 2025;44(1):52. doi:10.1186/s13046-025-03320-y
67. Kondo H, Tazawa H, Fujiwara T, et al. Osteosarcoma cell-derived CCL2 facilitates lung metastasis via accumulation of tumor-associated macrophages. *Cancer Immunol Immunother*. 2025;74(7):193. doi:10.1007/s00262-025-04051-x
68. Zhang N, Zhang S, Wu W, et al. Regorafenib inhibits migration, invasion, and vasculogenic mimicry of hepatocellular carcinoma via targeting ID1-mediated EMT. *Mol Carcinog*. 2021;60(2):151–163. doi:10.1002/mc.23279
69. Xuan X, Li Y, Huang C, Zhang Y. Regorafenib promotes antitumor progression in melanoma by reducing RRM2. *iScience*. 2024;27(10):110993. doi:10.1016/j.isci.2024.110993
70. Hsu FT, Sun CC, Wu CH, Lee YJ, Chiang CH, Wang WS. Regorafenib induces apoptosis and inhibits metastatic potential of human bladder carcinoma cells. *Anticancer Res*. 2017;37(9):4919–4926. doi:10.21873/anticancer.11901

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