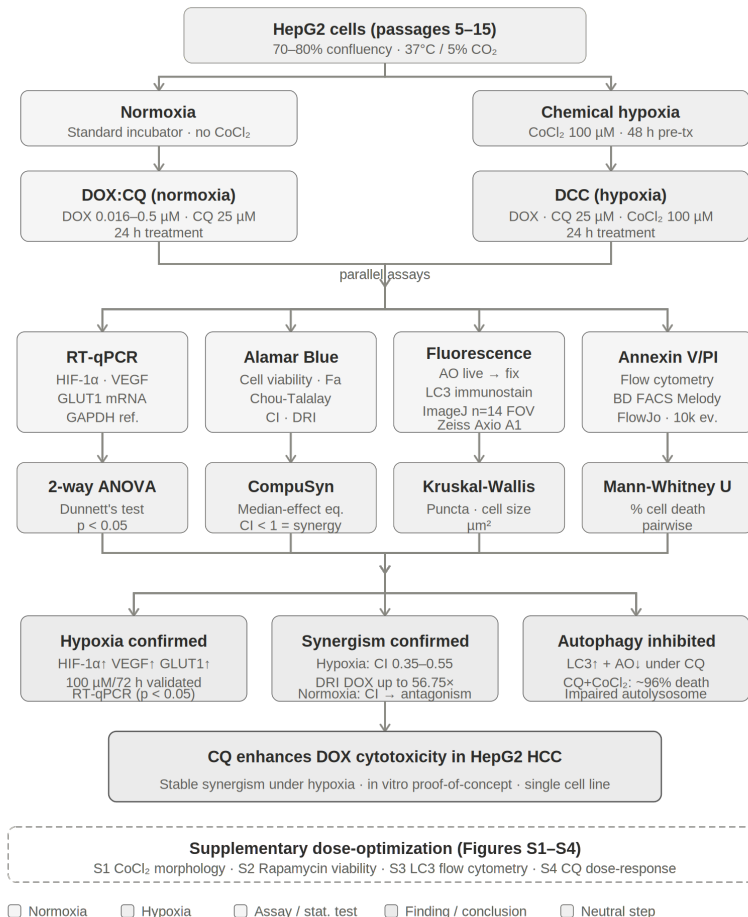


Supplementary

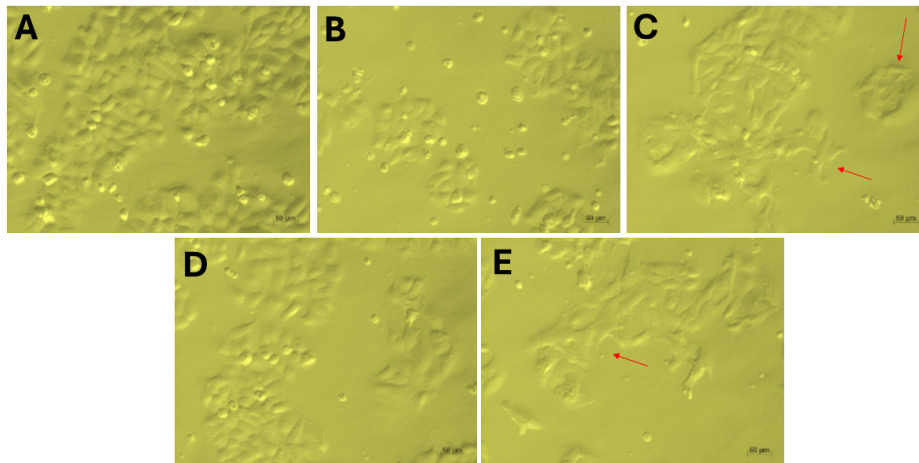
Nguyen, S. (2026). Chloroquine Enhances Doxorubicin Cytotoxicity in HepG2 Hepatocellular Carcinoma Cells: A Chou–Talalay Analysis under Normoxia and CoCl₂-Induced Hypoxia. *Biomedical Research and Therapy*, 13(9), 9017-9031.

<https://doi.org/10.15419/bmrat.v13i9.1103>

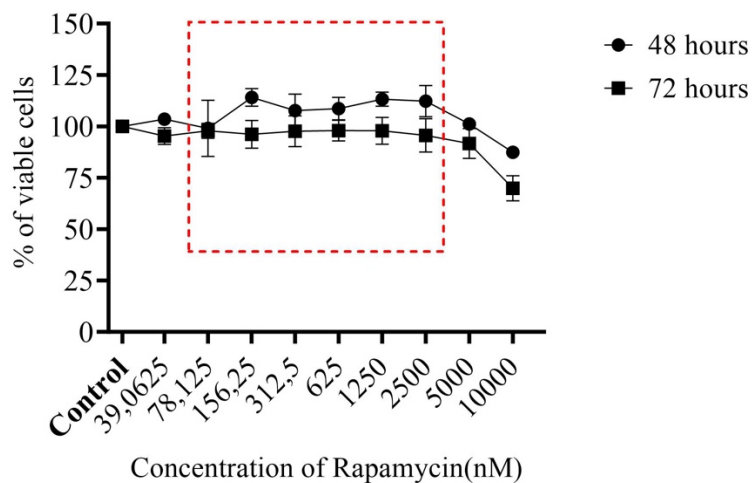


Supplementary Figure S0. Schematic overview of the experimental study design and methodological workflow. **Figure Legend:** Schematic diagram illustrating the complete experimental workflow utilized in this study. HepG2 cells were cultured under normoxic (21% O₂) or chemically simulated hypoxic (100 μM CoCl₂) conditions. Four parallel analytical pipelines were conducted: (1) chemical hypoxia transcriptional validation (RT-qPCR for HIF-1α, VEGF, GLUT1); (2) autophagic organelle remodeling assessment (sequential live-cell Acridine Orange staining and fixed-cell LC3A/B immunofluorescence microscopy); (3) quantitative apoptosis and cell death profiling (Annexin V–APC/7-AAD flow cytometry); and (4) pharmacological drug combination synergy and dose-reduction modeling (Chou–Talalay

median-effect equation and CompuSyn analysis). Key experimental endpoints and summary conclusions are indicated.

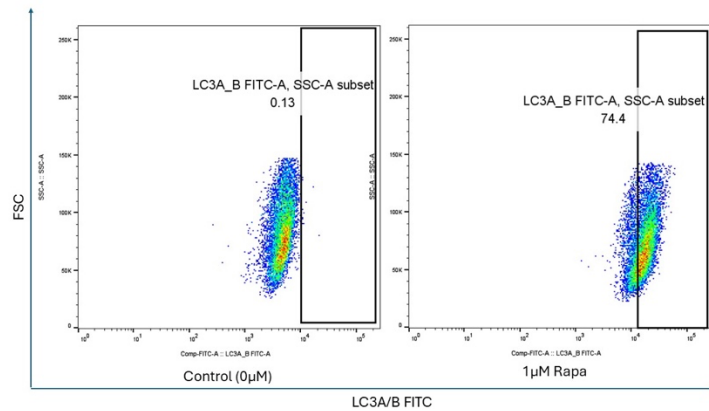


Supplementary Figure S1. Phase-contrast morphological alterations in HepG2 cells during CoCl₂ dose- and time-optimization screening. **Figure Legend:** Representative phase-contrast micrographs of HepG2 cells cultured in the presence of vehicle control or increasing concentrations of CoCl₂ (0, 50, and 100 μM) for 72 h and 96 h (panels A–E). Exposure to 100 μM CoCl₂ for 72 h induced characteristic morphological features of chemical hypoxia adaptation (cellular elongation, cytoplasmic coarseness) while maintaining >70% cell viability, establishing 100 μM / 72 h as the standardized hypoxic condition for all combination assays.

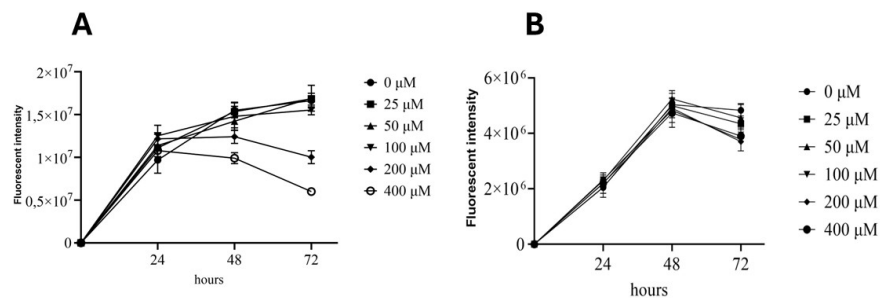


Supplementary Figure S2. Dose- and time-dependent cytotoxicity profile of rapamycin in HepG2 cells. **Figure Legend:** HepG2 cell viability assessed via Alamar Blue assay following treatment with rapamycin across a concentration range of 39.06–10,000 nM for 48 h and 72 h.

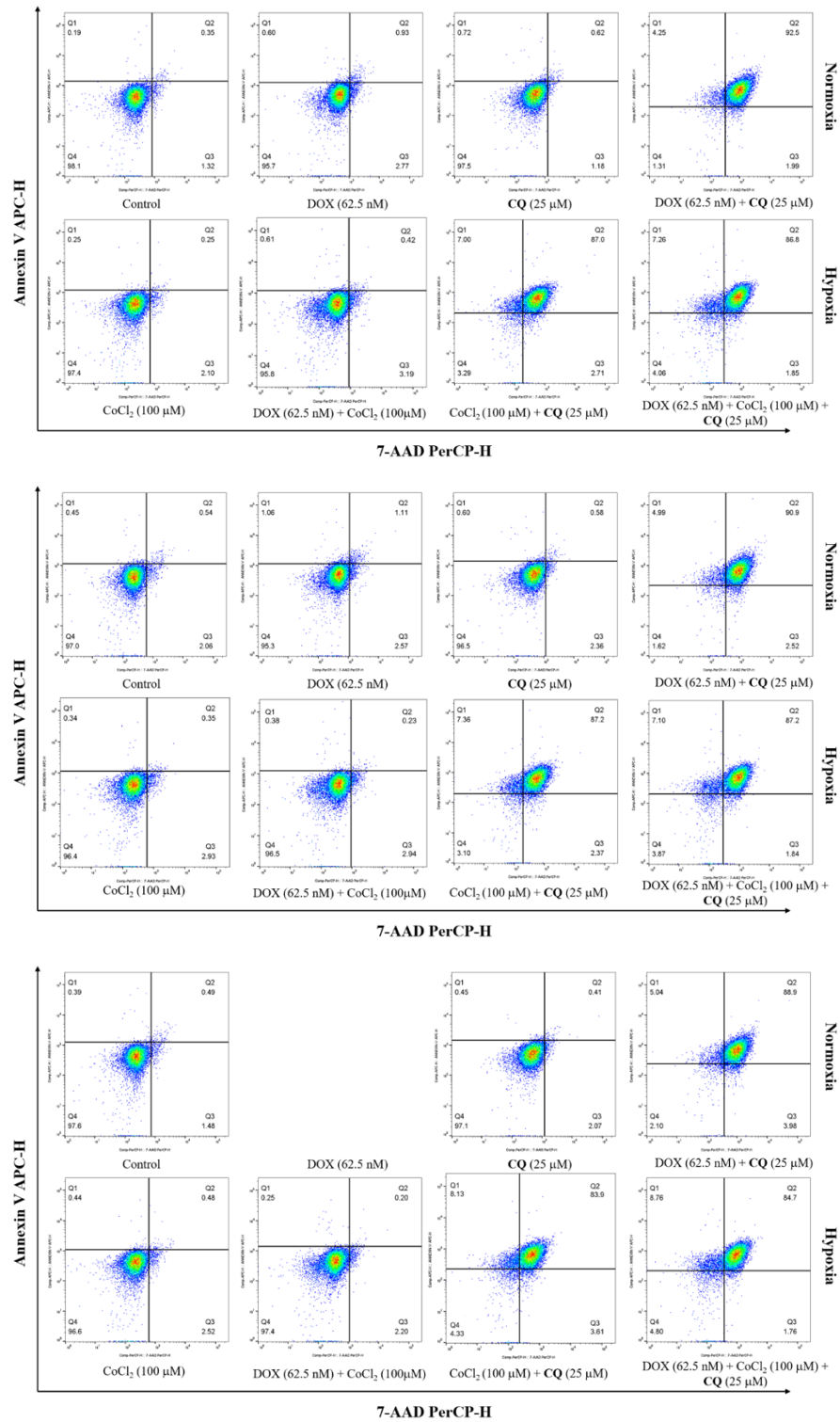
Data supported the selection of 1 μM rapamycin (24 h exposure) as the non-cytotoxic positive control concentration for autophagy induction assays.



Supplementary Figure S3. Flow cytometric validation of LC3A/B accumulation following positive control rapamycin treatment in HepG2 cells. **Figure Legend:** Flow cytometric histograms of permeabilized HepG2 cells immunostained with DyLight™ 488-conjugated anti-LC3A/B antibody following 24 h exposure to vehicle control or 1 μM rapamycin. Rapamycin treatment resulted in a substantial increase in LC3A/B-positive cells (from 0.13% in vehicle control to 74.4% in rapamycin-treated cells), confirming robust autophagosome induction at the selected working concentration.

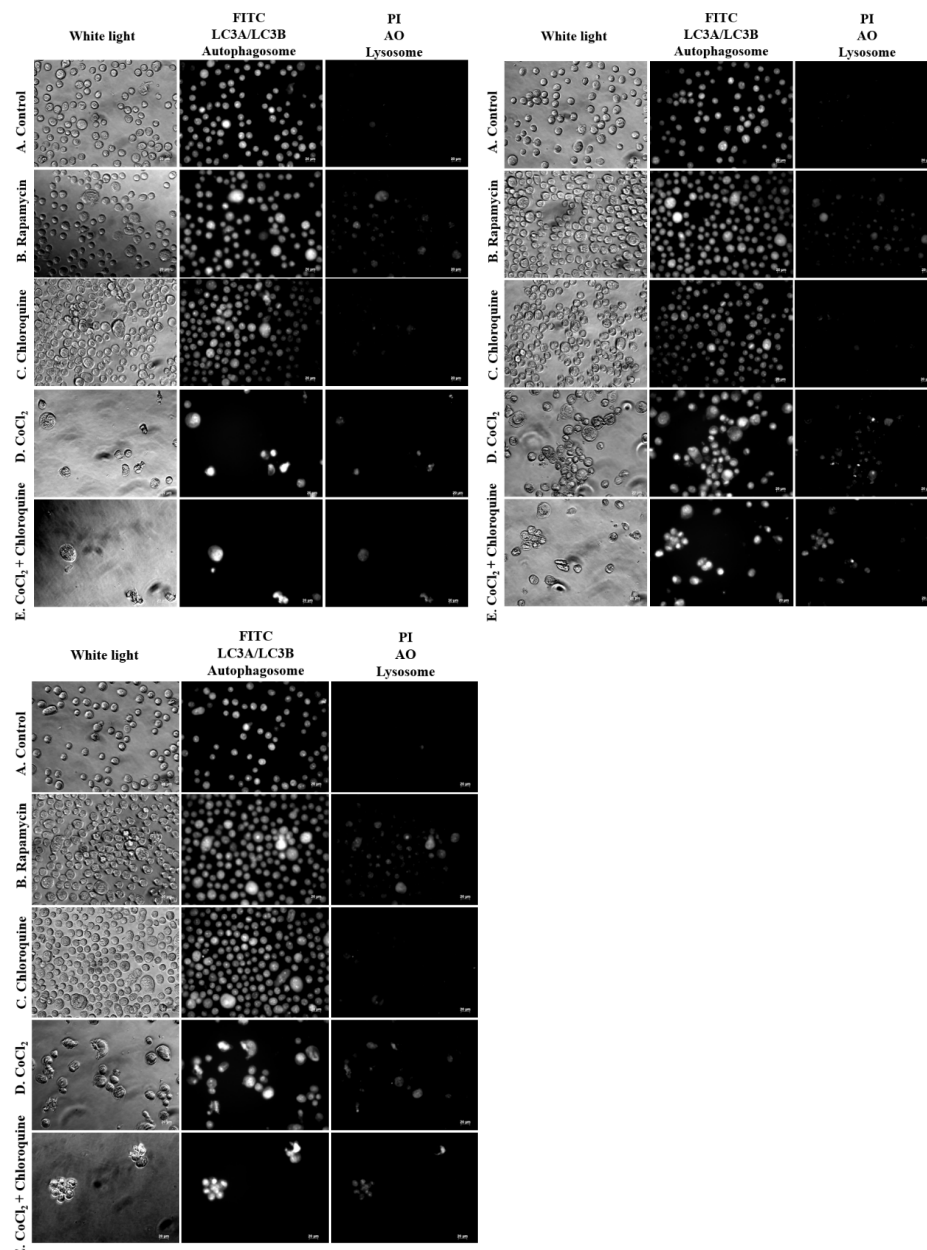


Supplementary Figure S4. Concentration-dependent viability screening of chloroquine under normoxic and CoCl_2 -induced hypoxic conditions. **Figure Legend:** HepG2 cell viability measured via Alamar Blue assay following exposure to chloroquine (CQ; 0–400 μM) for 48 h and 72 h under: (A) standard normoxic culture conditions, and (B) CoCl_2 -induced chemically hypoxic conditions (100 μM CoCl_2). At 25 μM , CQ exhibited minimal single-agent toxicity under normoxia while inducing measurable growth inhibition under hypoxia, supporting its selection as the standardized fixed concentration for combination synergy assays.



Supplementary Figure S5. Replicate Annexin V/7-AAD flow cytometric dot plots across all experimental treatment groups. Figure Legend: Complete panel of biparametric flow cytometry dot plots displaying Annexin V–APC (y-axis) vs. 7-AAD (x-axis) across three independent biological replicates for all eight experimental treatment conditions under normoxia

and chemical hypoxia (100 μ M CoCl₂, 72 h). Individual replicate numerical values for viable, early apoptotic, late apoptotic, and necrotic gates are compiled in Supplementary Table S5.



Supplementary Figure S6. Complete multi-field fluorescence microscopy panels of Acridine Orange and LC3A/B dual staining in HepG2 cells. **Figure Legend:** Multi-panel fluorescence micrographs displaying three independent replicate preparations (panels a–c) per treatment condition (vehicle control, 1 μ M rapamycin, 25 μ M CQ, 100 μ M CoCl₂, and 100 μ M CoCl₂ + 25 μ M CQ). Micrographs illustrate brightfield, FITC (LC3A/B; autophagosomes), Texas Red/long-pass (Acridine Orange; acidic autolysosomes/lysosomes), and merged co-localization channels. Scale bar = 20 μ m. Individual puncta counts from all quantified fields are documented in Supplementary Table S6.