



Article

Simultaneous Inhibition of MDM2 and XIAP by MX69 Induced Cell Cycle Arrest and Apoptosis in HUH7 and Hep3B Cell Lines

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Abstract

Genomic instability not only drives tumor initiation and progression but also cooperates with apoptosis resistance to promote therapeutic evasion in hepatocellular carcinoma (HCC). Activation of MDM2, a negative regulator of p53, together with XIAP overexpression, represents a critical axis underlying this resistance. Simultaneous targeting of MDM2 and XIAP by MX69, a small molecule inhibitor, may therefore offer a potent interventional strategy to suppress cell proliferation and enhance pro-apoptotic signaling in HCC in vitro models. To evaluate the effects of MX69, cell viability was assessed via CVDK-8, colony formation, and real-time cell analysis. Oxidative stress levels and DNA damage were examined using fluorescence imaging and comet assays, respectively, while mitochondrial membrane potential was monitored through JC-1 staining. Furthermore, flow cytometry was employed to quantify apoptotic cell death and cell cycle distribution, while Western blot analysis was used to characterize the expression of apoptosis-related proteins. In vitro cytotoxicity assays revealed that MX69 reduced the viability of HUH7 and Hep3B cells in a dose-dependent manner, suppressed colony formation, and exerted anti-proliferative effects in real-time proliferation assays. Cell viability and IC₅₀ values were evaluated using CVDK-8 and RTCA assays. Furthermore, MX69 induced oxidative stress and mitochondrial dysfunction, as evidenced by elevated ROS levels and loss of mitochondrial membrane potential. This was accompanied by significant DNA damage, detected by comet assay and γ -H2AX immunofluorescence, and G₀–G₁ cell cycle arrest. Moreover, MX69 triggered apoptotic cell death, demonstrating potent anticancer activity. Collectively, our findings identify MDM2/XIAP dual inhibition by MX69 as a promising therapeutic approach in HCC, with potential to overcome apoptosis resistance linked to genomic instability.

Keywords: HCC; MX69; MDM2/XIAP; DNA damage; apoptosis



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1. Introduction

Genomic instability is a critical hallmark of cancer, conferring the ability for tumor cells to develop characteristics which support initiation, progression and therapeutic resistance [1]. It is a common consequence of replicative stress resulting from DNA replication machinery errors or lack of response to DNA damage (DDR), and can crosstalk with numerous pathways important for cancer cell survival, such as immune avoidance, and resistance to apoptosis [2,3]. The most important characteristic of cancer is its resistance to apoptosis, and this continues to constitute an obstacle to the apoptosis of treated cells [4]. Therapeutic strategies targeting apoptosis resistance in cancer cells have been developed, but they still face many challenges [5]. For example, mouse double minute 2 (MDM2) oncoprotein functions as a critical negative regulator of p53 by promoting its ubiquitination

and proteasomal degradation [6]. Although MDM2 has traditionally been defined as a negative regulator of the p53 tumor suppressor, recent studies have revealed that this protein also exerts p53-independent functions in the DNA damage response. Severe genetic insults, such as DNA double-strand breaks (DSBs), require not only p53 activation but also the coordinated engagement of DNA repair machinery [7,8]. In this process, MDM2 has been shown to interact directly with the MRN complex (MRE11–RAD50–NBS1), a key sensor of DNA damage. In particular, MDM2 binding to NBS1 delays DSB repair, leading to increased chromosomal breaks and the development of genomic instability. Moreover, following DNA damage, MDM2 undergoes post-translational modifications including phosphorylation, ubiquitination, and changes in subcellular localization that facilitate p53 stabilization [9]. Collectively, these findings indicate that MDM2 functions not only as an E3 ubiquitin ligase regulating p53 degradation but also as a dynamic modulator of DNA repair and damage-sensing pathways [8]. X-linked inhibitor of apoptosis protein (XIAP) suppresses apoptosis by directly inhibiting caspase-3 and caspase-7, known as effector caspases [10]. Overexpression of MDM2 and XIAP has been reported to be associated with HCC [8,9], thus highlighting the central role of apoptosis regulation in liver tumorigenesis [10]. Targeting apoptotic resistance through strategies that combine DNA damage induction with inhibition of anti-apoptotic regulators is widely considered a promising approach. Therapeutics for HCC should also consider the liver specific metabolic context. The hepatic metabolism is the first pass of most chemotherapeutic agents; thus, enzymatic drug interactions are important causes of chemotherapy failure [11]. Sorafenib is still a standard first line therapy for advanced HCC; it is a multikinase inhibitor that targets anti-apoptotic pathways and exhibits anti-angiogenic and anti-proliferative activities [12]. However, drug resistance and significant side effects are quite common, yet their clinical benefit remains limited [12,13]. All of these factors highlight the critical need for new therapeutic approaches that increase efficacy and reduce toxicity. Recently, the MDM2 inhibitor MI-43 was shown to selectively kill lung cancer cells harboring wt p53 [14]. The study by Zheng et al. used the combination of the MDM2 inhibitor MI-219 and the XIAP inhibitor SM-164 in lung cancer cells. Acting alone, MI-219 selectively inhibited the growth of lung cancer cells containing wild-type (wt) p53 by inducing G1 or G2 arrest in a p53-dependent manner. MDM2 suppression had minimal effects on MI-219 induced growth suppression. Although MI-219 increased XIAP expression, blocking XIAP through SM-164, a Smac mimetic compound, did not selectively increase MI-219 cytotoxicity [15]. Cytotoxicity caused by MI-219 was not affected by MDM2 suppression or a XIAP inhibitor in combination. In this study, the dual action small molecule inhibitor MX69, which targets both MDM2 and XIAP, exhibits potent cytotoxicity, cell cycle arrest, and apoptosis [16,17]. Importantly, MX69 is selective for transformed cells over normal counterparts unlike classic inhibitors [16]. Nevertheless, its effectiveness in HCC has not been validated. In light of these challenges, we aim to investigate the effects of MX69 on cell proliferation, DNA damage, and apoptosis resistance in Hep3B and HUH7 cell lines. By integrating established liver cancer cell models our study seeks to provide mechanistic and translational insights into MDM2/XIAP dual inhibition as a potential therapeutic strategy for HCC.

2. Materials and Methods

2.1. Reagents

MX69 (HY-100892) was obtained from MedChemExpress (Monmouth Junction, NJ, USA). The Cell Cycle Analysis Kit used to determine the cell cycle was obtained from NutriCulture ECOTECH Biotechnology (Erzurum, Türkiye) (Catalog number: CCA50). FITC Annexin V Apoptosis Detection Kit with PI was purchased from BioLegend (Catalog number: 640914) (San Diego, CA, USA). Mitochondrial membrane potential measurements

were performed using JC-1 fluorescent, carbocyanine dye from Sigma (CAS number: 47729-63-5) (St Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and phosphate-buffered saline (PBS) were purchased from Gibco (Waltham, MA, USA). Primer antibodies (anti-GAPDH, Santa Cruz, CA, USA, sc-365062, 1:1000), Mcl-1 (Santa Cruz, sc 12756, 1:1000), BCL-2 (Santa Cruz, sc-7392, 1:1000), BAX (Abcam, Cambridge, UK, ab7977, 1:1000), H2AX (Santa Cruz, sc-517336, 1:1000), pATM (Santa Cruz, sc-47739, 1:1000), ATM (Santa Cruz, sc-377293, 1:1000), MDM2 (Santa Cruz, sc-965, 1:1000), XIAP (Santa Cruz, sc-55551, 1:1000), Caspase-3 (Santa Cruz, sc-56053, 1:1000), Caspase-9 (Santa Cruz, sc-56076, 1:1000), and secondary antibodies (Anti-mouse IgG-HRP, Jackson, West Grove, PA, USA, 115-035-166, 1:5000, Anti-rabbit IgG-HRP, sc-2357, 1:5000, Anti-goat IgG-HRP, sc-2020, 1:15,000) were used.

2.2. Mammalian Cell Source and Culture Conditions

In the cell culture steps of the study, liver cancer cell lines (Hep3B and HUH-7) were used, obtained from the cell line stock provided by BUKAG (Bingol University Cancer Research Group). To ensure optimal in vitro conditions, cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 64 µg/mL penicillin, and 100 µg/mL streptomycin in a humidified 37 °C cell culture incubator with 5% CO₂.

2.3. Determination of IC₅₀ Values with Cell Viability Determination Kit-8 (CVDK-8)

The anti-proliferative effect of MX69 was evaluated using the NutriCulture Cell Viability Determination Kit-8 (CVDK-8) (Boster, Pleasanton, CA, USA), which utilizes a water-soluble tetrazolium salt for colorimetric detection. Briefly, 3×10^3 cells were seeded per well in 100 µL medium in a 96-well plate and incubated for 24 h in a humidified CO₂ incubator at 37 °C. Following the initial incubation, the cells were treated with varying concentrations of MX69 for 24 and 48 h. After treatment, 10 µL of CVDK-8 reagent was added to each well, and the plates were further incubated for 1–4 h. The absorbance was subsequently measured at 450 nm using a microplate reader (Molecular Devices LLC, San Jose, CA, USA) [18].

2.4. Clonogenic Assay

Hep3B and HUH-7 cells were seeded into 6-well plates at densities of 1000 and 2000 cells per well, respectively. Following treatment, the cells were washed three times with PBS and maintained in a humidified incubator (37 °C, 5% CO₂) for 14 days to allow colony formation. The culture medium was refreshed every 3–4 days. The assay was terminated once colonies in the control group reached a minimum of 50 cells and before individual colonies became confluent. Subsequently, the cells were washed with PBS, fixed with a methanol–acetic acid (3:1) solution for 5 min, and stained with 0.5% crystal violet for 15 min. Clusters consisting of at least 50 cells were quantified as colonies, and the data were subjected to statistical analysis [19].

2.5. Real-Time Cell Analyses

The xCELLigence (ACEA Biosciences Inc., San Diego, CA, USA) imaging system was used for real-time cell analysis. Liver cancer cells were treated with concentrations determined by the CVDK-8 assay. Subsequently, the cells seeded on plates specially developed for the device were treated with the relevant agents and then placed in the device in a humid 37 °C cell culture incubator containing 5% CO₂. After programming, the cells were read for a period of time determined by pilot experiments for proliferation analysis (ACEA, San Diego, CA, USA) [20].

2.6. Determination of Intracellular Reactive Oxygen Species Level

Liver cancer cells were seeded in 6-well cell culture dishes with 2×10^5 cells/well. The next day, they were treated with the relevant agents and incubated in a CO₂ incubator. After incubation, the cells were washed three times with PBS and then incubated with 5 μ M DCFH-DA for 30 min in a carbon dioxide incubator. Then, the cells were washed again with PBS 3 times and cellular images were captured with an Olympus CKX41 Inverted Microscope (Olympus, Tokyo, Japan) with a 20 $\times$ objective [21]. Fluorometric analysis with DCFH-DA dye was performed as a different method to determine reactive oxygen species. Briefly, 25 μ M DCFH-DA solution was added to the medium containing the cells after the treatments and kept in a 37 °C CO₂ incubator for 45 min. At the end of the incubation period, the medium was removed from the wells and 1X wash solution was added to each well. The absorbance was then read at Ex 485 nm/Em 535 nm with a fluorescence spectrophotometer [22].

2.7. DNA Damage Detection (Single Cell Gel Electrophoresis-Comet)

Briefly, 1% high melting agarose (HMA) in phosphate buffer was dissolved in a microwave oven and after spreading on rubbed slides, it was kept at +4 degrees overnight. Cells were mixed with low melting agarose (100 μ L) at 40–42 °C and spread on slides coated with HMA after the respective agent treatments (1×10^4 cells). Immediately afterwards, the slides were kept at +4 °C and in the dark for 5 min to solidify the agar. The slides were then placed in cold lysis solution and kept in the dark at +4 °C for 45 min. After the lysis time expired, the preparations were electrophoresed in the electrophoresis tank at 25 V for 20 min. After electrophoresis, the preparations were washed with cold neutralization buffer for 3 $\times$ 5 min and stained with 40 μ L of 20 μ g/mL ethidium bromide/Redsafe and observed under fluorescence microscope. At least 50 cells from different photographs obtained from the preparations prepared for each group were analyzed using the ImageJ program (open comet) (Version 1.50i) [23].

2.8. Immunofluorescence

Hep3B and HUH-7 cell lines were grown on sterile coverslips in 24-well microplates. After the incubation period, defined MX69 dosages were individually applied to the cells. After the treatment period, the cells were fixed with 4% paraformaldehyde for 10 min. After that, the wells were washed with PBS and permeabilized with PBS, including 0.2% Triton X-100 on ice for 10 min. After the permeabilization, cells were blocked with 10% goat serum followed by incubation with anti-P-histone γ -H2AX (Genetex, Irvine, CA, USA) primary antibodies overnight at 4 °C. Cells were washed and incubated with secondary antibodies conjugated with Alexa Fluor 594 and 488 (Invitrogen, Carlsbad, CA, USA). Finally, sealed sections were examined and photographed with a fluorescence microscope (EVOS FL, Invitrogen). Merged images were generated by overlaying with GIMP 2.10.8 software [24].

2.9. Mitochondria Membrane Potential ($\Delta\Psi_m$) Measurement

JC-1 Stock solution (1.5 mM) in DMSO was prepared according to the manufacturer's instructions. After the incubation period, the cells were treated with MX69. Following a 48-h treatment with MX69, cells were incubated with a 5.0 μ M JC-1 working solution in PBS for 30 min in the dark at 37 °C. After incubation, the cells were washed with PBS, and images were captured using a fluorescence microscope (Olympus, Tokyo, Japan) using both red and green channels. The mitochondrial membrane potential ($\Delta\Psi_m$) was quantified by calculating the ratio of red (J-aggregates) to green (J-monomers) fluorescence intensity. Total count and total area were analyzed with ImageJ (Version 1.50i). Color thresholding was used for area calculation [25].

2.10. Flow Cytometry Cell Cycle Analysis

Cells were seeded in 6-well plates and treated with MX69 for specified durations (24, 48, and 72 h). Following treatment, cells were harvested, washed with PBS, and fixed in ice-cold 70% ethanol for at least 1 h at -20°C . Fixed cells were then washed again and stained with a solution containing $5\text{ }\mu\text{g/mL}$ propidium iodide (PI) for 30 min in the dark. The resulting samples were then analyzed using CytExpert 2.5 software to determine the proportion of cells in each phase of the cell cycle based on their DNA content [26].

2.11. Determination of Cell Death by Flow Cytometry

The evaluation of apoptosis was conducted using the Annexin V-FITC Apoptosis Detection Kit. Initially, the cells were cultured in 100 mm dishes at a density of 4×10^5 cells/Petri and exposed to the appropriate agents after a 48-h incubation period. After incubation, cells were treated with $10\text{ }\mu\text{L}$ of Annexin V-FITC and $10\text{ }\mu\text{L}$ of PI and incubated for 20 min at room temperature in the dark. Then, binding buffer ($500\text{ }\mu\text{L}$) was added. The fluorescence intensity of the cells was detected by flow cytometry (Beckman Coulter, Cytoflex, Brea, CA, USA) [27].

2.12. Determination of Protein Expression Levels by Immunoblotting Method

To determine the expression levels of relevant proteins, total protein contents were extracted and the expression levels of the target proteins were assessed through immunoblotting. Following the application of MX69, cell culture dishes underwent two washes with cold 1X PBS buffer. Total protein extraction was carried out using a standard lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, NP-40 0.5%, (*v/v*), 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and a protease inhibitor cocktail (Complete EDTA-free). The cells, collected and suspended in 1X PBS buffer, were centrifuged at 5000 rpm for 10 min, followed by resuspension of the resulting pellet in lysis buffer. Suspension was facilitated using 1 mL syringes to ensure homogenization and protein extraction. Subsequently, the samples were centrifuged at 15,000 rpm 4°C for 10 min, and the supernatant was transferred to a new microcentrifuge tube. Total protein concentrations were determined using the Bradford method, and protein samples were separated by SDS-PAGE gel (4–20%) at appropriate concentrations. The separated proteins were then transferred to a PVDF membrane and incubated in blocking buffer (5% BSA or Skim Milk Powder). Following blocking, the membranes were incubated with the relevant primary antibody overnight at 4°C . After washing the membrane with 1X T-BST, it was incubated with an HRP-labelled secondary antibody (Anti-mouse IgG-HRP: 1:5000, Anti-rabbit IgG-HRP: 1:5000, Anti-goat IgG-HRP: 1:15,000) corresponding to the primary antibody used. Another wash with 1X T-BST was performed before imaging using an ECL kit based on the chemiluminescence method. The band intensities were analyzed densitometrically using ImageJ (Version 1.50i) [28].

2.13. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (Prism 10, GraphPad Software Inc., La Jolla, CA, USA). Data were presented as mean $\pm$ standard deviation (SD) of at least three experiments. Comparisons between the control and treatment groups were made using one-way ANOVA and Tukey's multiple comparison tests. The level of statistical significance was considered based on the *p* value (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Statistical analysis was performed with GraphPad Prism 10.0 (GraphPad Software, Inc.) software.

3. Results

3.1. MX69 Significantly Reduced Cell Viability, Colony Formation, and Proliferation

Our study first investigated the cytotoxic effect of MX69 on liver cancer cell lines. Incubation was performed using MX69 in HUH-7 and Hep3B cell lines at treatment doses of 0, 1, 1.5, 2, 5, 10, 20, and 40 μM . The 24-h MX69 treatment (Figure 1A,B) reduced cell viability. Conversely, 48-h MX69 treatment (Figure 1A,B) clearly demonstrated a higher mortality rate compared to the 24-h treatment, even though the same doses were used. Based on the experimental results, the final dose range to be used in all experimental sets of the project was set to values below the IC_{50} value, and treatment durations were set to be 48 h. To investigate the colony-forming abilities of MX69, clonogenic assays were performed 14 days after treatment with MX69 (0, 0.25, 0.5, 1, 2.5, and 5 μM) for 48 h in both HUH-7 and Hep3B cell lines. It was previously determined that MX69 could reduce the clonogenic capacity of the cells due to its anticancer activity in HUH-7 and Hep3B cells (Figure 1C). A dose-dependent change was noted in both cell lines compared to the control group. The real-time xCELLigence system was used for the profiling of MX69. Different concentrations of MX69 produced a dose-response pattern similar to that of CVDK-8 in both HUH-7 and Hep3B cell lines (Figure 1D). A dose-dependent antiproliferative effect was observed in both cell lines at 48 h of treatment.

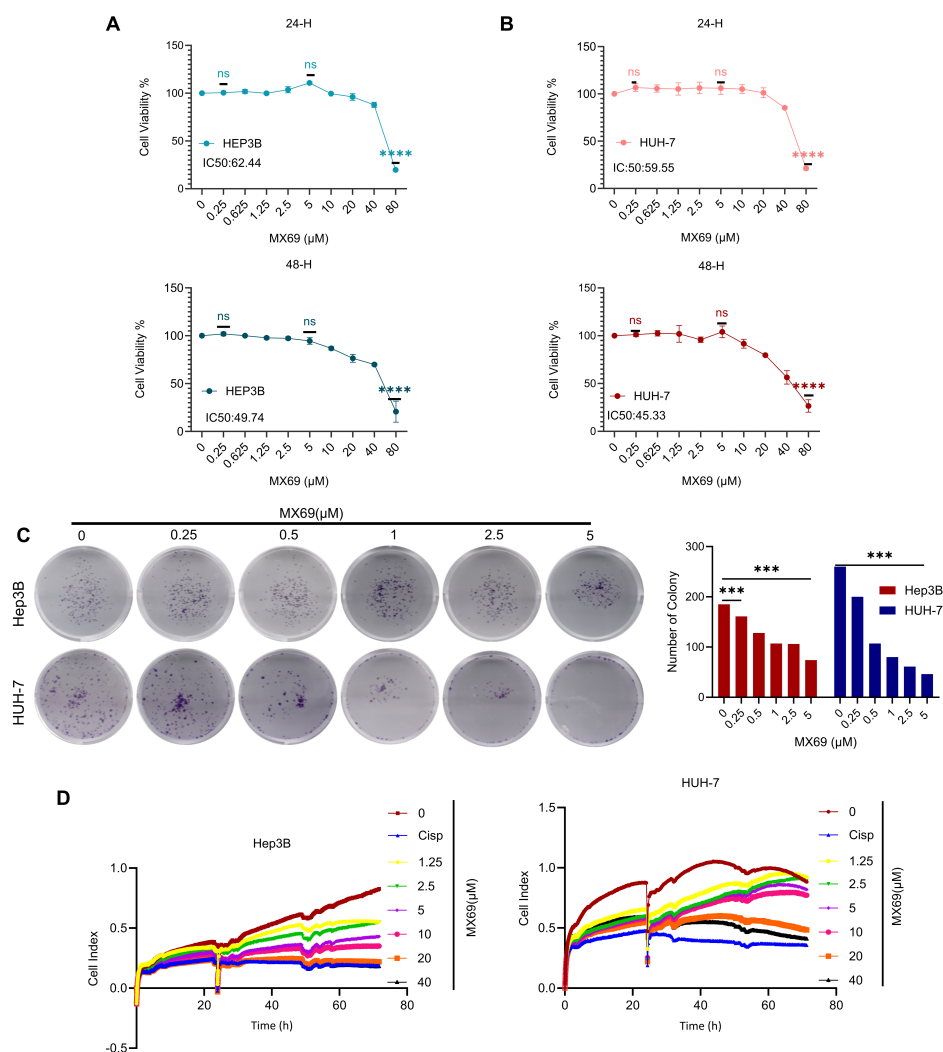


Figure 1. MX69 reduces viability, proliferation, and colony formation in Hep3B and HUH-7 cells. (A,B) HUH-7 and Hep3B cells were treated with MX69 (0.25, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80 μM) or a

vehicle control (DMSO, 1%) for 24–48 h, respectively. The control group was evaluated against the MX69 treatment groups. Cell viability was assessed using the CVDK-8 (Cell Viability Determination Kit-8) assay. Cell viability and IC₅₀ values (24 h: MX69: Hep3B, 62.44 μ M; HUH-7, 59.55 μ M; 48 h: MX69: Hep3B, 49.74 μ M; HUH-7, 45.33 μ M) were evaluated using the CVDK-8 assay. Other applied experimental doses were determined using dose ranges below the IC₅₀ value. (C) HUH-7 and Hep3B cells were treated with MX69, medium was replaced, and colony growth was noticed at Day 14. Three independent experiments were conducted to assess colony formation, and representative figures are presented. The histogram indicates the quantity of colony formation. Cisplatin as a positive control. (D) xCELLigence proliferation assay of cells in response to MX69. Hep3B and HUH-7 cells were seeded in 8-well E-plates for xCELLigence assay monitoring of impedance (cell index). All experiments were repeated at least three times, and data are presented as mean $\pm$ SD. One-way ANOVA and Tukey's multiple comparison test were used in statistical analyses (** $p < 0.001$ and **** $p < 0.0001$; ns = not significant) compared to untreated cells.

3.2. Effects of MX69 on Intracellular Reactive Oxygen Species Level in Hepatocellular Cancer Cell Lines

To determine the effect of MX69 treatment on intracellular ROS accumulation in HUH-7 and Hep3B cell lines, measurements were first performed using a fluorescence spectrophotometer and fluorescent DCFH-DA dye. MX69 increased the amount of ROS in a statistically significant way compared to the control group in a dose-dependent manner in HUH-7 and Hep3B cell lines (Figure 2A,B). Following 48 h of MX69 treatment (0, 2.5, 20 and 40 μ M), all cells were incubated with DCFH-DA for 30 min and fluorescent images were obtained using an immunofluorescence microscope. Statistically significant increases in ROS levels were noted in the treated Hep3B and HUH-7 cells (Figure 2C–E).

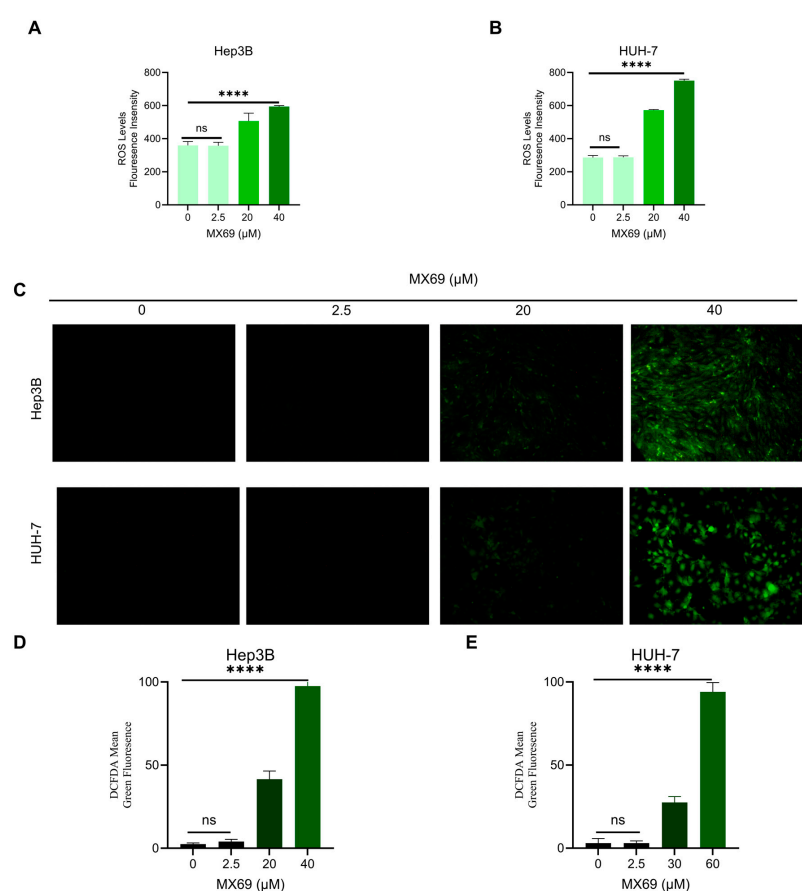


Figure 2. The effect of MX69 on intracellular ROS levels in HCC cell lines. (A,B) Spectrophotometric measurement of fluorescence intensity and (C–E) fluorescence microscopy images (original

magnification 20×) showing the increase in ROS in cells treated with MX69 at various concentrations for 48 h. In both methodologies, samples were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) at a concentration of 15 μ M for 30 min at 37 °C. Data represent averages $\pm$ SD of three independent experiments (**** $p < 0.0001$; ns = not significant).

3.3. XIAP/MDM2 Pharmacological Inhibitor MX69 Induces MMP Decline in HUH-7 and Hep3B Cell Lines

We examined whether MDM2/XIAP inhibition would lead to changes in mitochondrial membrane potential ($\Delta\Psi$ m). Following 48 h of treatment in Hep3B and HUH-7 cell lines, fluorescence microscopy revealed that the mitochondrial membrane potential remained intact in the control group, as evidenced by red fluorescence (JC-1 aggregates). In contrast, MX69 treatment induced a dose-dependent transition to intense green fluorescence (JC-1 monomers), indicating mitochondrial depolarization (Figure 3A–C). The significant decrease in the red-to-green fluorescence intensity ratio confirmed the loss of mitochondrial membrane potential in both cell lines compared to the control group.

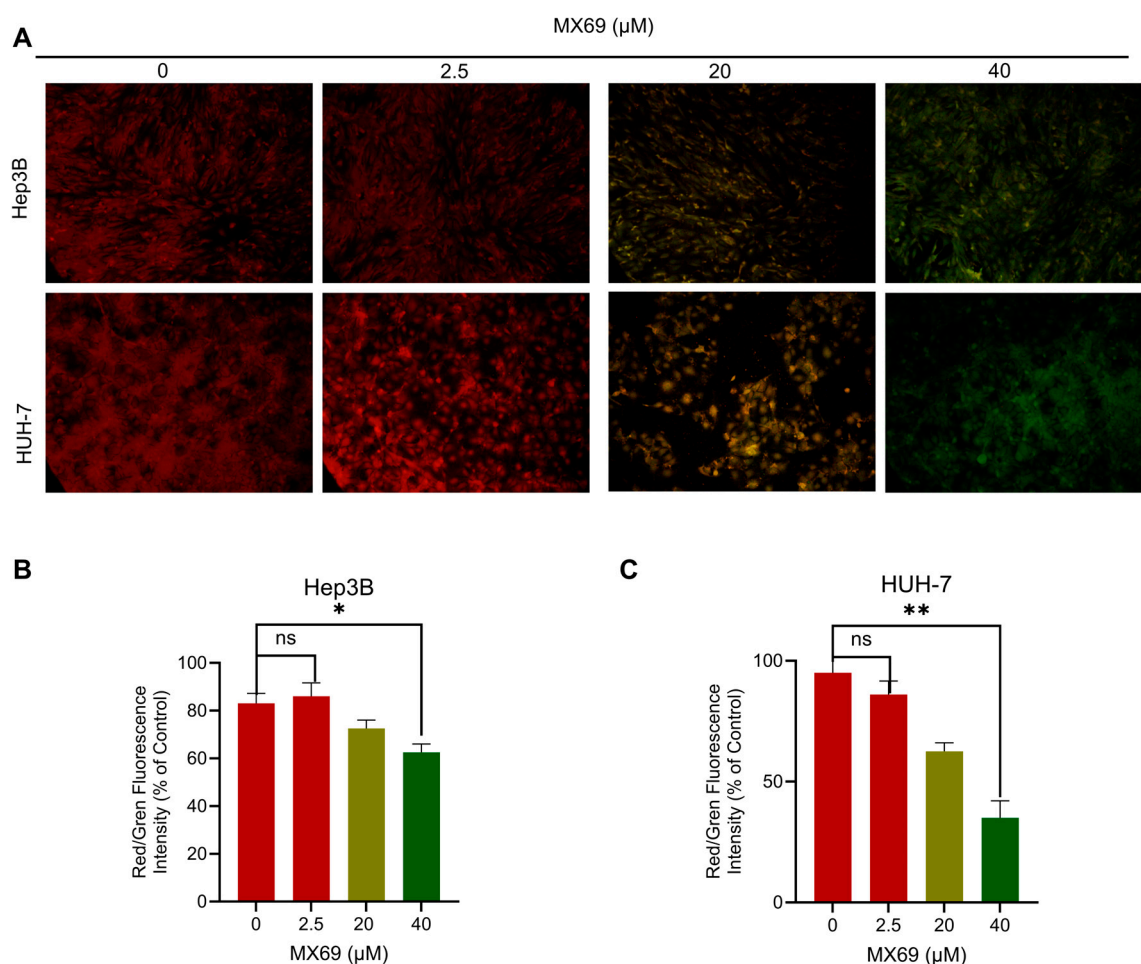


Figure 3. MX69 disrupted MMP in the Hep3B and HUH-7 cell lines. (A–C) The images (original magnification 20×) acquired through JC-1 staining, together with the quantification of red and green fluorescence intensity, were analyzed for the Hep3B and HUH-7 cell lines after treatment with MX69 at various concentrations over a period of 48 h. In the lower part of the panel, the ratio of red to green fluorescence intensity is quantified and displayed as histograms. Results are presented as the mean $\pm$ SD and reflect three independent experiments. $n = 3$. One-way ANOVA and Tukey's multiple comparison test were used to calculate statistical significance, and the calculations were performed using GraphPad Prism 10.0 software (GraphPad Software, Inc.) (* $p < 0.05$; ** $p < 0.01$; ns = not significant).

3.4. DNA Damage Is Induced by MX69 in Hep3B and HUH-7 Cells

To determine the genotoxic effects of MX69, comet assays and immunofluorescence analyses were performed on Hep3B and HUH-7 cells. It was observed that 48 h of MX69 treatment resulted in DNA damage in a dose-dependent manner, with the damage proportional to the lengths of the tails. A significant difference was noted in the 20 μ M and 40 μ M treatment groups compared to the control group (Figure 4A–C). The inhibition of MDM2/XIAP activity and the subsequent formation of DSB were found to be statistically significant in the treatment groups. In contrast, no changes were detected in ATM protein levels. A dose-dependent increase in pATM and H2AX proteins was observed (Figure 4D,E).

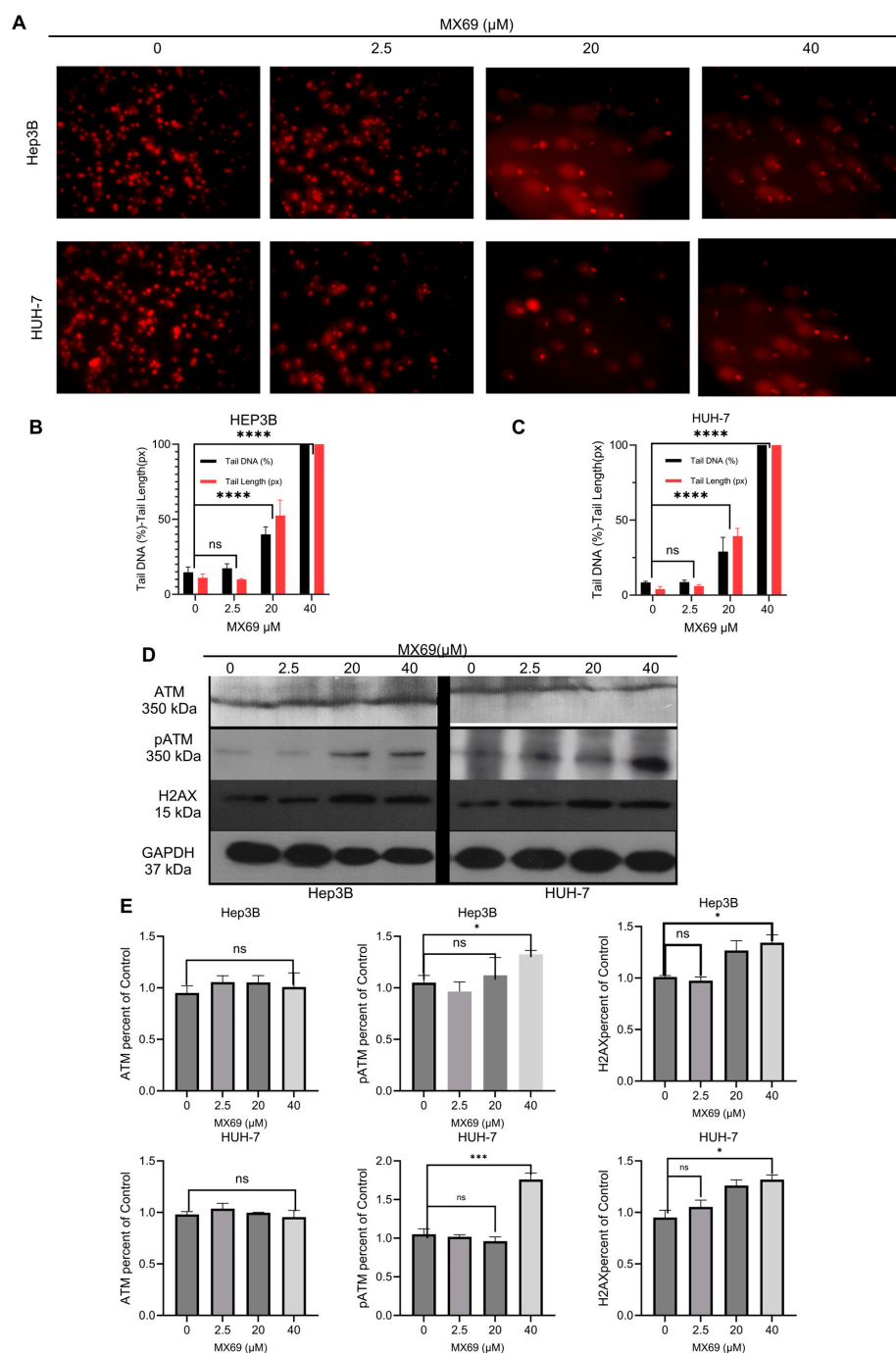


Figure 4. Comet assays were conducted on Hep3B and HUH-7 cell lines. (A–C) This assay assesses DNA fragmentation by measuring the length and percentage of tail DNA generated from Hep3B and

HUH-7 cell nuclei post-treatment with various doses of MX69 (2.5–20 and 40 μM , with a duration of 48 h in all instances), followed by alkali treatment and single-cell electrophoresis. Images (original magnification 20 $\times$) representing comets are displayed. The length and percentage of tail DNA in the comet assay were quantified and analyzed using ImageJ software. Results are presented as the mean $\pm$ SD and reflect three independent experiments ($n = 3$). (D,E) Determination of the effect of MX69 on DNA damage markers in Hep3B and HUH-7 cell lines by the Western blot method (ATM, p-ATM, H2AX, with GAPDH used as a loading control). One-way ANOVA and Tukey's multiple comparison test were used to calculate statistical significance, and the calculations were performed using GraphPad Prism 10.0 software (GraphPad Software, Inc.) (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$, ns = not significant).

Immunofluorescence images of histone H2AX phosphorylation in HUH-7 and Hep3B cells stained with γH2AX are shown in Figure 5A,B. Treatment with MX69 for 48 h resulted in a time-dependent induction of γH2AX foci. MX69 treatment induced focus formation and increased the percentage of γH2AX -positive cells. The data in Figure 5 show that MX69 exhibited different concentration and time-dependent effects ($p < 0.0001$) on γH2AX focus formation in cells.

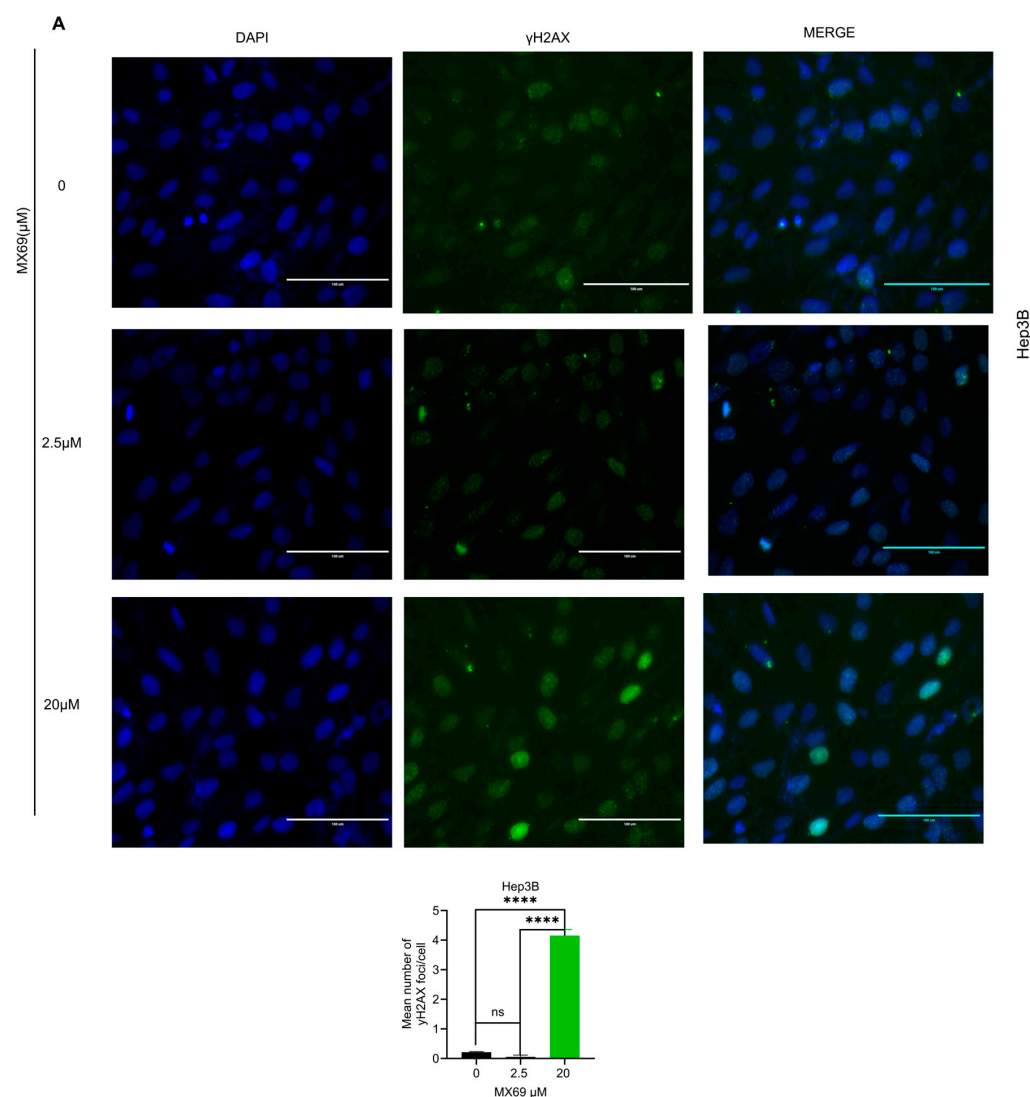


Figure 5. Cont.

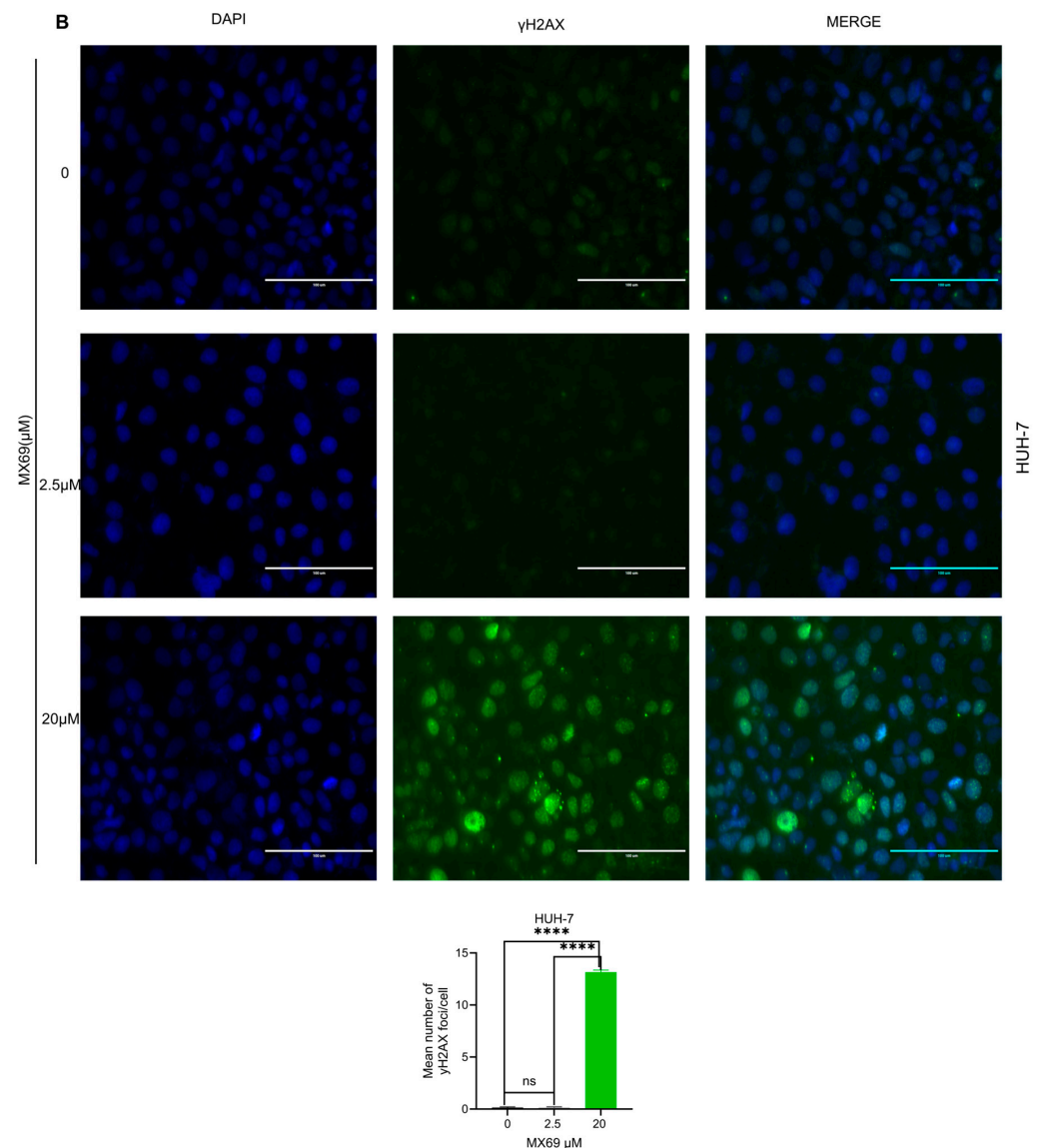


Figure 5. DNA damage in liver cancer cells was examined by immunofluorescence staining. (A,B) The cells were treated with MX69 (2.5 and 20 μM), and the cells were examined for γ-H2AX foci formation. γH2AX foci (original magnification 20×) were quantified as foci per nucleus for each dose of γH2AX foci number per nucleus from three separate experiments, wherein nuclei cells were scored per dose (**** $p < 0.0001$; ns = not significant).

3.5. Effects of MX69 on Cell Cycle Regulation in Hep3B and HUH-7 Cells

Propidium iodide (PI) staining and flow cytometry were used to analyze changes in the cell cycle. To determine the effect of MX69 alone on cell cycle arrest in both cell lines, cells were treated for 48 h (Figure 6A–C). PI staining results showed that the treatment significantly increased the number of cells in the G0/G1 phase while decreasing the number of cells in the S phase. These findings suggest that the single treatment induced cell cycle arrest at the G0/G1 checkpoint.

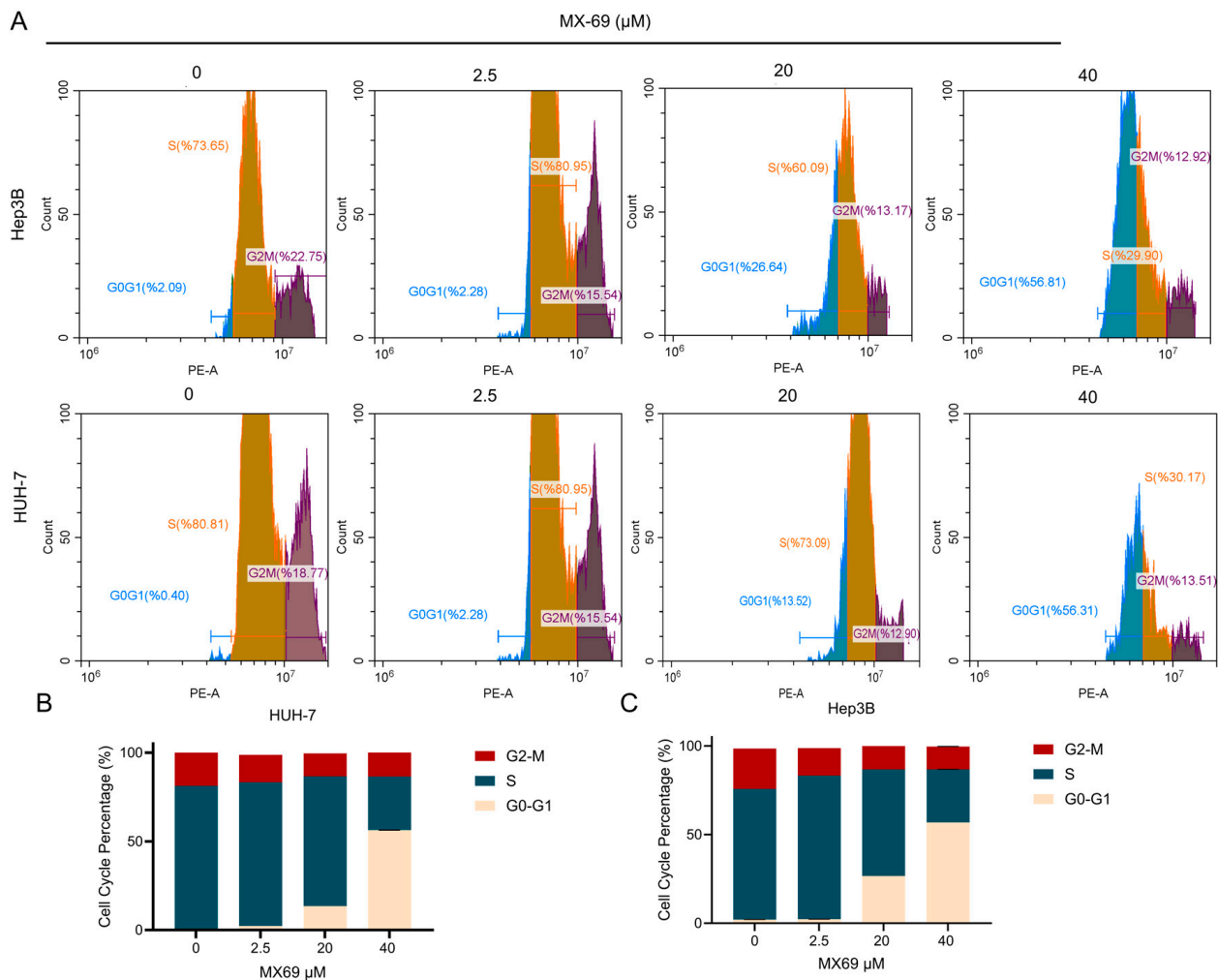


Figure 6. MX69 arrests cell cycle arrest in liver cancer cell lines. The investigation of the cell cycle in response to MX69 was conducted using flow cytometry. (A) Hep3B and HUH-7 cell lines were subjected to various concentrations of MX69 for a duration of 48 h. (B,C) The resulting histograms illustrate the distribution of cell populations. Treatment with MX69 resulted in an increased accumulation of Hep3B and HUH-7 cells within the G0/G1 phase, while a corresponding decrease in the number of cells in the S phase was observed when compared to the untreated control (results are presented as the mean of three independent experiments, $n = 3$).

3.6. MX69 Induces Cell Death in Hep3B and HUH-7 Cells

To investigate whether MX69 induces apoptosis in Hep3B and HUH-7 cells, cells were treated with 2.5, 20, and 40 μ M MX69 for 48 h, followed by Annexin V-FITC/propidium iodide (PI) double staining. Flow cytometric analysis revealed a dose-dependent increase in both early and late apoptotic cell populations in both cell lines compared to the control group. A similar trend was observed in late apoptotic cells, where MX69 treatment significantly enhanced the proportion of Annexin V and PI-positive cells. While the proportion of necrotic cells varied depending on the treatment concentrations, a significant induction of apoptosis was particularly evident in the HUH-7 cell line. These findings, detailed in Figure 7A–C, indicate that MX69 inhibits cell growth primarily by inducing apoptosis. The pro-apoptotic effect of MX69 was further confirmed by Western blotting. Treatment led to increased levels of pro-apoptotic proteins, including BAX, Caspase-3, and Caspase-9. Conversely, a dose-dependent decrease in the expression of anti-apoptotic proteins such as XIAP, MCL-1, and MDM2 was recorded (Figure 7D,E).

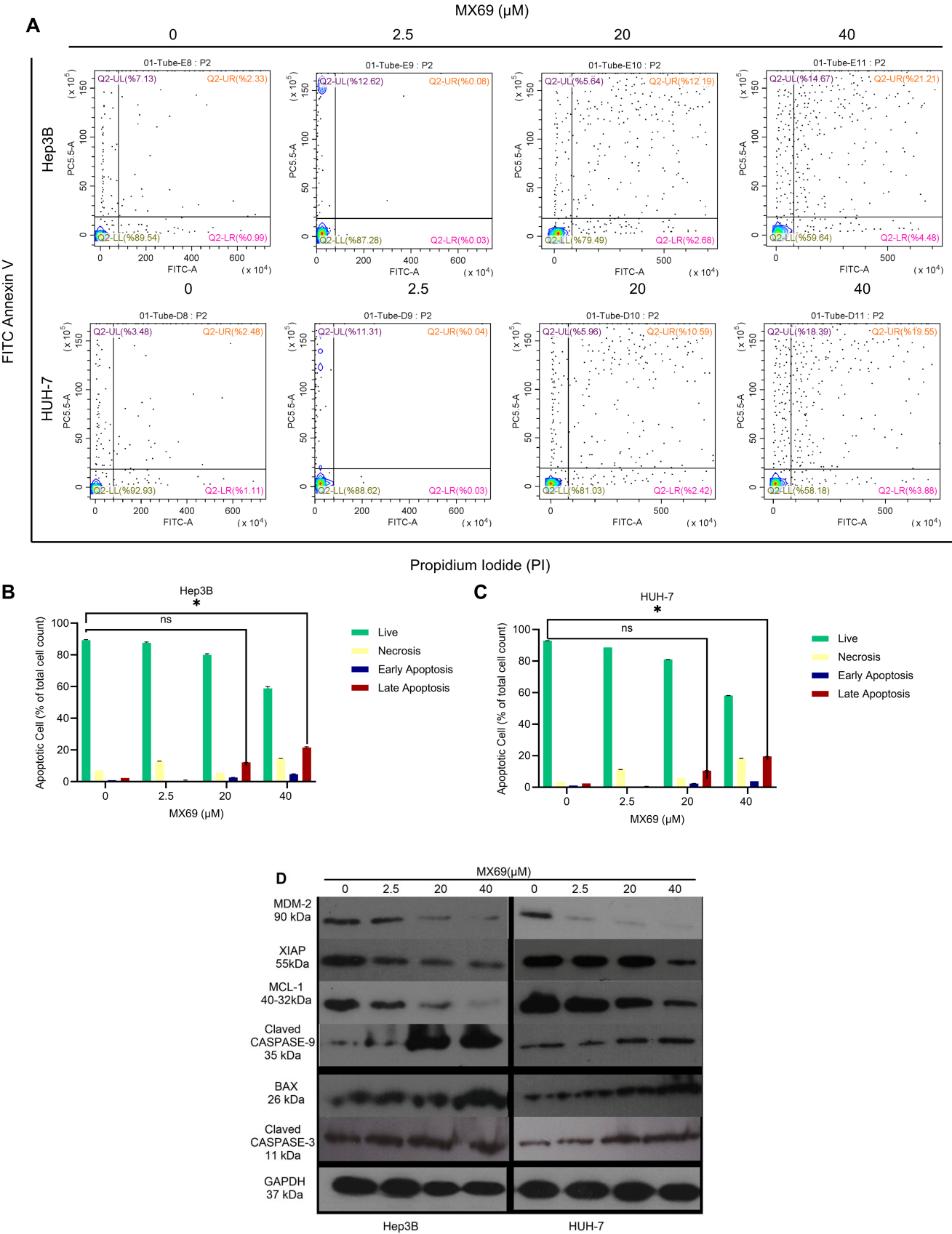


Figure 7. Cont.

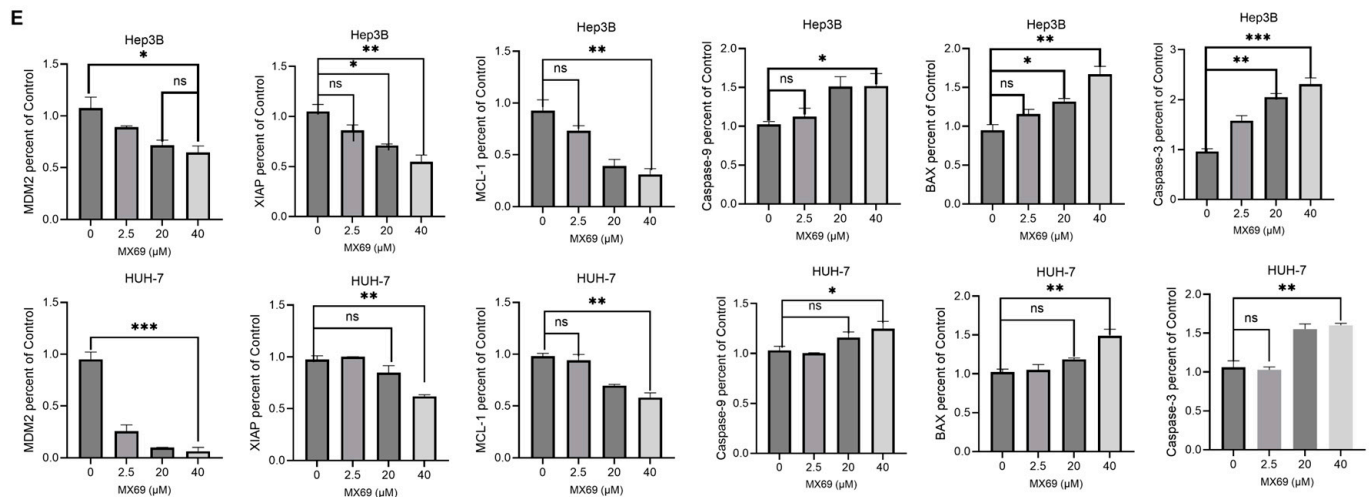


Figure 7. MX69 induces the apoptosis of Hep3B and HUH-7 cell lines. The induction of apoptosis in response to MX69 was evaluated by flow cytometry. Hep3B and HUH-7 cells were exposed to increasing concentrations of MX69 for 48 h, after which apoptotic rates were quantified via annexin V/propidium iodide staining. (A) MX69 induces apoptosis in liver cancer (Hep3B and HUH-7). Q1-LL (Control), Q1-UL (Necrosis), Q1-UR (Late Apoptosis), Q1-LR (Early Apoptosis). (B,C) Histograms of apoptotic cells (% of total cell count). The values are expressed as the mean $\pm$ SD ($n = 3$ /group). (D,E) Determination of the effect of MX69 on apoptosis markers in HUH-7 and Hep3B cell lines by the Western blot method. Apoptosis markers were MDM2, XIAP, MCL-1, BAX, Caspase-3, and Caspase-9, with GAPDH used as a loading control. The values are expressed as the mean $\pm$ SD ($n = 3$ /group). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test to evaluate differences between treatment doses within each cell line. Calculations were performed using GraphPad Prism 10.0 software (GraphPad Software, Inc.) (* $p < 0.01$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant).

4. Discussion

Liver cancer is divided into various subtypes, primarily HCC, which accounts for 75–85% of cases. It is reported to be the sixth most common cancer and the third leading cause of cancer-related death worldwide [29]. HCC is one of the most common malignancies of the digestive system and poses a serious health threat [30]. The protooncoprotein MDM2 is overexpressed in many tumors, including HCC, and plays a role in tumor development through its p53-dependent/independent functions [31,32]. Additionally, anti-apoptotic proteins (Bcl-xl, Mcl-1, c-IAP1, XIAP, and survivin) are expressed at high levels in HCC, promoting proliferation. Although XIAP, in particular, plays a role in carcinogenesis, progression, and metastasis, its effects in HCC have not been fully elucidated [30,33,34]. Despite advances, effective options for the treatment of advanced HCC remain limited, and new therapeutic approaches are urgently needed [35]. In recent years, inhibitors targeting MDM2 have gained prominence, and molecules such as MX69 and nutlin-3 have been studied specifically for suppressing MDM2 expression. By blocking the interaction between MDM2 and XIAP, MX69 disrupts MDM2 autoubiquitination, reduces XIAP expression, and contributes to the suppression of apoptosis and proliferation [34]. MX69 binds to the RING domain of MDM2 and also interacts with the IRES region of XIAP mRNA, inhibiting RING activity and reducing XIAP protein levels. Thus, it both activates p53 and disrupts XIAP, preventing apoptosis. Dual-targeting MX69 requires lower doses compared to single target inhibitors to achieve effective antitumor activity, which helps reduce toxicity. In this study, we aimed to eliminate cell survival by targeting the increased MDM2 and XIAP expression in HCC cells with MX69. In our initial cell viability assays, MX69 demonstrated potent anti-proliferative effects, dose-dependently reducing the viability of Hep3B cells at 24 and

48 h; consistent results were observed in HUH-7 cells under the same treatment conditions. Our results are consistent with those obtained by On et al. in their study on triple-negative breast cancer (TNBC), where they examined the pharmacological inhibition of MDM2. Three different MDM2 inhibitors used in TNBC cell lines were shown to effectively reduce cell viability during 72 h of treatment [31]. On the other hand, in a study by Lubing and his team, a cell viability assay was used to determine the cytotoxic effects of MX69 on a healthy cell line and a neuroblastoma cell line. The results showed that it reduced cell viability in a dose-dependent manner in both cell lines [16]. In another experiment conducted on liver cancer cell lines, colony formation was performed to determine the colony-forming ability of the cells. A 48-h experiment with MX69 (0, 0.25, 0.5, 1, 2.5, and 5 μ M) in the Hep3B and HUH-7 cell lines demonstrated a dose-dependent decrease in the colony forming ability of the cells. Albadari et al.'s study also treated melanoma and prostate cancer cells with MDM2/XIAP inhibitors (0, 1, 5, or 10 μ M). Following treatment, the colony-forming ability of the cells decreased as the dose increased in both cell lines [32]. Real-time cell analysis (RTCA) revealed that cell proliferation was reduced in a dose-dependent manner in both cell lines. Previous studies support our findings that MDM2 inhibition induces organoid cell death. For example, Luo et al. observed a decrease in gastric SRCC organoid numbers after MDM2 inhibitor treatment [36], whereas XIAP-deficient intestinal organoids exhibited increased sensitivity to TNF/ZVAD-induced death compared to wild-type controls [37].

MX69 treatment led to a significant, dose-dependent increase in ROS levels in both HUH-7 and Hep3B cells, with the 20 and 40 μ M doses showing statistical significance, which was further confirmed by enhanced DCFH-DA fluorescence and microscopy at 40 μ M. Consistent with our findings, previous studies have shown that XIAP inhibition increases ROS levels in a dose-dependent manner. Ding et al. reported elevated ROS in HeLa cells following 6-h treatment with XIAP inhibitors (0–30 μ M) using DCFH-DA assays [38], while Munoz et al. observed increased ROS in MCF-7 and U373 cells treated with XIAP inhibitors and stained with DCFH-DA [39]. Our findings suggest that ROS buildup is not just a byproduct of cell death, but an active trigger. The fact that ROS levels increased alongside mitochondrial damage indicates that oxidative stress is an upstream event that helps drive the cells toward apoptosis.

In line with our results, MX69 induced dose-dependent genotoxic effects in HUH-7 and Hep3B cells, as evidenced by increases in both tail DNA and tail length in comet assays following 48-h treatment. These findings highlight the capacity of MX69 to induce DNA damage in liver cancer cells. To confirm DNA damage, expression levels of H2AX and ATM proteins were examined in a Western blot experiment. While pATM expression was increased in liver cells treated with MX69 in a dose-dependent manner, no change in ATM expression was observed, as expected. H2AX protein expression was found to be increased in a dose-dependent manner in both cell lines. Interestingly, in contrast, Soares et al. reported that treatment of ROS lymphocytes with an MDM2 inhibitor (7–21 μ M) for 48 h did not elicit measurable increases in tail length, unlike the positive control etoposide [40], suggesting that MX69's geno-toxicity may be context- or cell type-dependent. Concordantly, Cheng et al. demonstrated that targeting survivin with the inhibitor YM155 enhanced DNA damage in breast cancer cells, as evidenced by increased comet tail length [41]. To further assess DNA damage, we employed immunofluorescence and observed MX69-induced γ H2AX foci formation in both liver cancer cell lines that was markedly increased compared to the controls.

Flow cytometry revealed that MX69 induced a dose-dependent G0/G1 arrest in both Hep3B and HUH-7 cells, accompanied by a reduction in S and G2/M populations, highlighting its impact on cell cycle progression. Our findings on MX69 induced cell cycle alterations are consistent with the results of the initial preclinical study by Gu et al. [16].

Unlike Nutlin-3, which requires functional p53, MX69 maintains its efficacy regardless of p53 status due to its dual-targeting of MDM2 and XIAP. This dual mechanism allows MX69 to bypass common p53 mutations, offering a more versatile therapeutic option for a broader range of HCC patients. Annexin V staining revealed that MX69 triggered dose-dependent cell death in both HUH-7 and Hep3B cells, characterized by reduced viable populations and increased necrosis, early, and late apoptosis. Notably, at 40 μ M, the majority of cells shifted toward late apoptosis, underscoring the potent pro-apoptotic activity of MX69. The pro-apoptotic effects observed in our study align with the initial report on MX69, reinforcing its conserved mechanism of inducing apoptosis across cancer models. Consistent with its pro-apoptotic activity, MX69 treatment for 48 h increased Bax, Caspase-3, and Caspase-9 levels in both HUH-7 and Hep3B cells while concomitantly reducing the anti-apoptotic proteins Mcl-1, XIAP, and MDM2 in a dose-dependent manner, underscoring a shift toward apoptotic signaling. In line with our findings, McManus and colleagues reported that shRNA-mediated inhibition of XIAP in MDA-MB-231 breast cancer cells failed to sufficiently reduce the expression of the anti-apoptotic protein Mcl-1 while concomitantly increasing the pro-apoptotic factor Bax [42]. Our findings suggest that the potent effect of MX69 is mediated by ROS induction, DNA damage, and mitochondrial dysfunction, ultimately culminating in programmed cell death, as illustrated in the schematic representation (Figure 8).

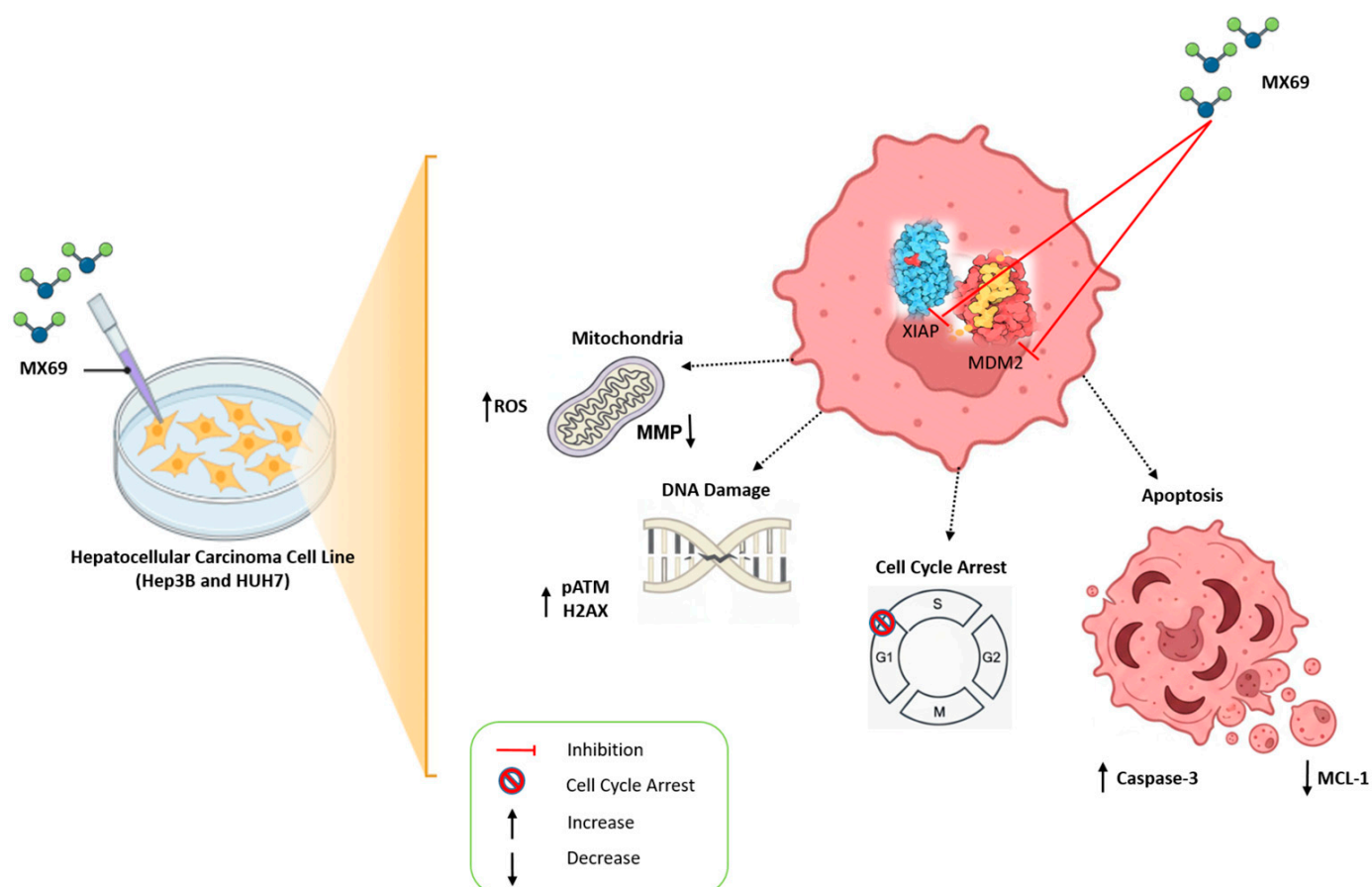


Figure 8. Schematic representation of the mechanistic pathways induced by MX69 in HCC cells (Hep3B and HUH-7 cell lines). MX69 treatment triggers significant DNA damage and ROS production, causing the mitochondria to lose their membrane potential. It simultaneously halts the cell cycle and triggers apoptotic cell death.

5. Conclusions

In conclusion, our study demonstrates MX69 as a potent dual inhibitor of MDM2 and XIAP in HCC. By targeting both proteins simultaneously, MX69 offers a robust therapeutic strategy that maintains its efficacy regardless of p53 status, effectively bypassing a major clinical hurdle where traditional inhibitors like Nutlin-3 often fail. While these findings are limited by the in vitro conditions, they provide a strong basis for further investigation to translate MX69 into clinical use. Future research involving in vivo models and combination therapies will be essential to fully harness its potential for enhancing treatment outcomes in HCC patients.

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Abbreviations

The following abbreviations are used in this manuscript:

HCC	Hepatocellular Carcinoma
MDM2	Murine Double Minute 2
XIAP	X-Linked Inhibitor Of Apoptosis
DDR	DNA Damage Response
DMSO	Dimethyl Sulfoxide
RTCA	Real-Time Cell Analyzer
FBS	Fetal Bovine Serum
CI	Cell Index
NMA	Normal-Melting-Point Agarose
LMA	Low-Melting-Point Agarose
PVDF	Polyvinylidene Difluoride
IC ₅₀	Half-Maximal Inhibitory Concentration
MMP	Mitochondrial Membrane Potential
PBS	Phosphate Buffered Saline
CVDK-8	Cell Viability Determination Kit-8

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