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Azithromycin's Cytotoxic Effects and CASP8 Expression Modulation in HT-29 Colorectal Cancer Cells In Vitro

Marzieh Hoveida [1], Hadi Habibollahi [2*], Mehrnaz Ahmad Sharbafi [3]

[1] Department of Biology, Ra.C., Islamic Azad University, Rasht, Iran. E-mail: marry71m2@gmail.com, ha.habibollahi@iau.ac.ir, m.ahmadsharbafi@iau.ac.ir

[*] *Corresponding author*, Department of Biology, Ra.C., Islamic Azad University, Rasht, Iran. E-mail: ha.habibollahi@iau.ac.ir

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Colorectal cancer has been a leading cause of cancer deaths worldwide. This demands novel therapeutic approaches to combat resistance and the side effects of current conventional treatments. In this study, we explored azithromycin to determine if it could be repurposed to interfere with cytotoxicity in HT-29 CRC cells. We also examined CASP8 gene expression, an important signal-transducing actor in extrinsic apoptotic pathways. The cells were cultured in DMEM with FBS and antibiotics. Treatments involved azithromycin concentrations from 10 to 1000 µg/ml. After 24 hours, cytotoxicity was assessed by MTT assays. CASP8 expression was examined by qRT-PCR, using GAPDH as the reference gene. Statistical analyses included one-way ANOVA with Dunnett post-hoc test, ROC curve plotting for viability, and unpaired t-test for gene expression. Dose-dependent cytotoxicity was observed. Cell viability decreased from 100% in controls to about 2-3% at concentrations ≥100 µg/ml ($P < 0.0001$). This decline plateaued as concentrations increased, with perfect discriminatory power (ROC AUC = 1.000). Transcript levels of CASP8 showed no significant difference between groups ($P = 0.5106$). These findings indicate azithromycin's potential antiproliferative effects on CRC, possibly through CASP8-independent mechanisms such as inhibition of autophagy. However, mRNA data alone cannot confirm pathway involvement. This finding justifies further in vivo, mechanistic, and protein-level investigations for azithromycin's potential repositioning as an adjunctive treatment.

Introduction

Colorectal cancer (CRC) remains a very common and deadly malignancy. It accounts for nearly 10% of all cancer diagnoses and is the second most common cause of cancer-related death (Lu et al., 2021). In CRC, unregulated proliferation occurs in the colonic epithelium. A cascade of genetic and epigenetic changes can cause adenomatous polyps to progress to invasive adenocarcinoma (El Zarif et al., 2022). The growing global burden of CRC calls for newer therapeutic approaches. This need arises mainly from chemotherapy resistance and harsh side effects of conventional treatments (Asadi et al., 2018). Recent reports highlight repurposing old medicines like antibiotics to target cancer pathways. This approach may offer a cheaper way to boost therapeutic efficacy (Mohi-Ud-Din et al., 2023). Macrolide antibiotics such as azithromycin attract interest for their non-antimicrobial effects. They may directly influence processes such as apoptosis and autophagy, thus inhibiting tumors

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even without the classic inflammation seen with many cancer therapies (Fiorillo et al., 2019). In this study, we tested the cytotoxic potential of azithromycin against colorectal cancer cells. We aimed to further onco-therapeutic efforts and improve patient outcomes in this field.

Azithromycin is a derivative of erythromycin that inhibits bacterial protein synthesis via the binding of the 50S ribosomal subunit; while its antibiotic action on bacteria is well studied, its non-antibacterial eventualities for eukaryotic cells have received greater interest (Heidary et al., 2022). What makes azithromycin interesting is its immunomodulatory and anti-inflammatory effects, with its accumulation in phagocytes and fibroblasts brain to rather high tissue concentrations (Firth & Prathapan, 2020). In cancer therapy, this drug has been repurposed to interrupt cancer cell metabolism and signaling pathways, including the blockade of autophagy, which supports tumors under stress (Takano et al., 2023). Previous studies have demonstrated that azithromycin inhibits the proliferation of many cancers. It synergistically promotes the cytotoxicity of vincristine by upregulating the expression of death receptors in cervical and gastric cancer cells (Zhou et al., 2012). In turn, in lung cancer models, it amplifies the effect of DNA-damaging agents by inducing lysosomal membrane permeabilization (Toriyama et al., 2021). On account of this, azithromycin could involve varied mechanisms, involving mitochondrial dysfunction, upregulation of oxidative stress, and suppression of vascular endothelial growth factor receptor 2, thus highlighting it as a potential candidate for CRC therapy (Li et al., 2017; Zhang & Xu, 2023). The key therapeutic parameters evaluated here comprise cytotoxicity changes over time and gene expression variations in *CASP8* in CRC. Cytotoxicity is assessed through cell viability tests like MTT, thereby determining the capacity of any compound in killing target cells the death of cells being a reflection of any disturbances cast on the metabolic activity and membrane integrity by any test item (Athamneh et al., 2017). In a CRC case, changes in cell viability inversely correlate with apoptosis, wherein programmed cell death eliminates cancerous cells (Wong, 2011). *CASP8* encodes caspase-8, which functions as an initiator caspase in the extrinsic pathway of apoptosis and leads to DNA fragmentation via the activation of several downstream effectors upon ligation of death receptors (Nadendla et al., 2025). Dysregulation of *CASP8* expression has been linked to CRC progression; for instance, inactivating mutations of *CASP8* are associated with tumor survival and drug resistance, whereas the upregulation of *CASP8* was proven to be a mechanism of increased sensitivity to treatments (Kim et al., 2003). Agents increase caspase expressions in CRC cell lines like HT-29, thus leading to apoptosis, i.e., phycocyanin or nisin (Asoudeh-Fard et al., 2024; Hosseini et al., 2020). Henceforth, *CASP8* is involved in the cross-talk of apoptosis with other types of death and therefore serves as an investigative biomarker for the evaluation of novel therapies (Jiang et al., 2021). There exists a rather large void in the literature about specific mechanisms relating to azithromycin and CRC, in particular with the modulation of apoptosis genes such as *CASP8*. Some studies have indicated azithromycin to be cytotoxic in lung and breast cancers through inhibition of autophagy or by targeting mitochondria, but only a handful of studies have been carried out on its dose-dependent cytotoxicity on CRC cell lines or gene expression profiles (Semba et al., 2023; Takano et al., 2023). For example, much of the literature has ignored potential interactions of azithromycin with caspase pathways in colorectal models, with a number of conflicting views on the drug's apoptosis-promoting or apoptosis-inhibiting role (Favaloro et al., 2012; Vogler et al., 2025). More importantly, most of the tested cell types and exposure durations vary widely across laboratories, thus limiting the interpretation of long-term transcriptional changes or in the unresolved debates on the role of non-apoptotic pathways in resistance to CRC (Contadini et al., 2023; Müller et al., 2020). Our study ventures to correct these deficiencies by exploring the roles of azithromycin on the HT-29 cell line and thus filling the essential gap in repurposed drug research for CRC (Mohapatra & Ray, 2024). An extensive attempt has been made to study the cytotoxicity of azithromycin in colorectal cancer cells, HT-29, and its effects on *CASP8* gene expression, seeking to determine whether azithromycin exerts a dose-dependent cytotoxic effect and whether it modulates the transcription of *CASP8* in the CRC cells.

Material and Methods

Cell culture and cell cytotoxicity assessment

The HT29 cell line from Royan Research Center, Isfahan, Iran, was cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. To prevent contamination, antibiotics were added: 100 U/mL penicillin G and 100 µg/mL streptomycin. Additionally, 2 g/L $NaHCO_3$ was added to regulate pH balance. The cell lines were grown at 37 ºC in a CO_2 incubator set to 5% CO_2 and 95% humidity. Cytotoxicity of azithromycin was assessed in five independent biological replicates, each with technical triplicates (n=5 per group for analysis). Azithromycin powder was dissolved directly in culture medium (no additional solvent used, to avoid potential interactions). Cells were seeded into 96-well plates at 5,000 cells per well and incubated at 37°C with 5% CO_2 and 95% humidity for 24 hours. Cells were then treated with azithromycin at concentrations of 10, 50, 100, 250, 500, or 1000 µg/ml, alongside untreated controls (final solvent concentration: 0%, as no solvent was used). After 24 hours, the medium was replaced with fresh medium containing 0.5 mg/mL MTT (Sigma) dissolved in PBS. Plates were incubated in the dark for 4 hours at 37°C. Formazan crystals were solubilized with 200 µL DMSO per well, followed by 25 µL Sorensen's glycine buffer. Absorbance was read at 570 nm with a reference wavelength of 630 nm using an ELx800 Microplate Absorbance Reader (Bio-Tek Instruments). Background subtraction was performed using blank wells (medium only). Viability data were normalized per plate to the control wells.

Total RNA extraction and cDNA synthesis

The cultured cells were trypsinized in specific flasks and then collected by centrifugation at 1,320g for 3 minutes at 37°C. Next, total RNA was extracted using a commercially available kit (Sinaclon, Iran), following the Trizol method for both treatment and control cell lines. The efficiency and effectiveness of this RNA extraction technique were assessed using nanodrop analysis. Additionally, the extracted RNA purity was verified using a spectrophotometer (Thermoscientific, Wilmington, DE, USA) at 260–280 nm, and the quality of RNA extraction was validated through agarose gel electrophoresis at a 0.5% concentration. When the pure extracted RNA was ready, cDNA was synthesized from it using oligo dT primers following the protocol of the Sinaclon reverse transcriptase kit. The synthesized cDNA was promptly prepared for use in Real Time PCR, while the remaining cDNA was stored at -70°C.

Real-time PCR

Here, the Real-time PCR technique was used to compare the amount and amount of variation of the *CASP8* gene expression in treated and control samples, while *GAPDH* gene expression was used as a quantitative data normalization internal control. In detail, the expression level of the *CASP8* gene in the samples was assessed by quantitative Real-time PCR (qRT-PCR) technique utilizing the Syber Green-I (Sinaclon, Iran) with MIC Real-Time PCR Cycler (BMS, South Korea) in triplicate based on the procedure working with the manufacturer. For conducting the Real-time PCR, *CASP8* (Genbank accession: NM_033355.4, bp 1090-1190) and *GAPDH* (Genbank accession: NM_001357943.2, bp 256-431) primers were used. For *CASP8*, a 100 base pair and for *GAPDH* a 175 base pair amplification size was generated in a 25 µl reaction mixture consisting of: 5 moles of the forward and reverse PCR primers of *CASP8* (5'TCATGGACCACAGTAACATGGA3', 5'AGTGAACTGAGATGTCAGCTCAT3' respectively) or for *GAPDH* (5'GGAGCGAGATCCCTCCAAAAT3', 5'GGCTGTTGTCATACTTCTCATGG3' respectively), 2X PCR Master Mix Syber Green I and 2µl of the cDNA was used. Whereas the conditions and modalities of Real-time PCR reactions were assessed through analyzing standard samples in triplicate. A 5-Fold serial volume of cDNA obtained from the HT29 cell line was analyzed as a sample for full expression of the CASP8 gene. The protocol used for the real-time PCR reaction was as follows: an initial denaturation step at 95°C for 5 minutes and then 40 cycles of 95°C denaturation for 30 seconds, 57°C annealing for 30 seconds, and 72°C extension for 30 seconds. Finally, amplicons were analyzed with a melting curve analysis from 72°C to 95°C. Changes in the amounts of *CASP8* expression between control and HT29 cells treated with Azithromycin, normalized to GAPDH mRNA amounts, were compared using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

For statistical analysis, GraphPad Prism 10.6.0 (desktop version) was used to evaluate the effects of Azithromycin on HT29 colon cancer cell

viability and *Casp8* transcript levels. Cell viability was assessed using a one-way ANOVA to compare multiple Azithromycin concentrations, followed by Dunnett's post-hoc test to determine significant differences from the control. Homogeneity of variances was confirmed with Brown-Forsythe and Bartlett's tests. Receiver Operating Characteristic (ROC) curve analysis was performed to evaluate the discriminatory power of the viability assay at 10 µg/ml. For *CASP8* transcript levels, an unpaired t-test with Welch's correction was conducted to compare treatment and control groups, with an F-test used to verify comparable variances between groups.

Results

The investigation elucidated the effect of Azithromycin on the viability of the HT29 colon cancer cell line, revealing a significant cytotoxicity effect. The treatment of Azithromycin at 10 to 1000 µg/ml significantly inhibited cell viability compared to untreated cell control, where the decrease in viability appeared to be drug dose-dependent to a specific dose, and began to plateau at higher doses. Overall, these data suggest that Azithromycin decreased HT29 cellular proliferation, as seen in the viability results and supported by statistical evaluation analyses, including ANOVA and the ROC curve. Implications of these data suggest that Azithromycin may have therapeutic utility in colorectal cancer models; however, further mechanistic research is needed.

The viability of cells was evaluated using a typical assay, with results reported as a percentage of viability in relation to the control (100%). By 10 µg/ml of Azithromycin injection, the mean viability was reduced to 30.16%, with further reductions at higher concentrations being: 50 µg/ml (6.903%), 100 µg/ml (3.382%), 250 µg/ml (2.189%), and 500 µg/ml (2.129%). At the highest concentration of 1000 µg/ml, the mean viability was 2.985%. The data exhibit an initial dramatic decline in viability when comparing control to 10 µg/ml, with additional decreases occurring until 100 µg/ml, at which point the viability was approximately consistent from 250 to 1000 µg/ml (mean viability remained at <3%). The bar graph in Figure 1 shows these mean values with error bars indicating the standard deviation, with evidence of a dose response. The statistical analysis demonstrated that these observations were significantly important. The one-way ANOVA, based on the assumption of normality of variance across groups, indicated that the treatment groups showed highly significant differences in mean values ($F(6, 28) = 120.5$, $P < 0.0001$, $R^2 = 0.9964$). Homogeneity of variances was assessed and confirmed using both the Brown-Forsythe ($F(6, 28) = 0.8412$, $p = 0.5498$) and Bartlett's tests, supporting the validity of the one-way ANOVA. After confirming normality and homogeneity of variance assumptions, Dunnett's multiple comparisons test, which compared each concentration rather than the significance between all pairs to represent the control, showed significant differences at all concentrations (Adjusted $P < 0.0001$ for each concentration) with observed mean reductions from the mean of the control group ranging from 69.84% for 10 µg/ml to 97.87% for 500 µg/ml. The detailed one-way ANOVA and post-hoc results are reported in Table 1.

To assess the ability of the viability assay to discriminate between control and treated cells (at 10 µg/ml), a ROC curve was created with an area under the curve (AUC) of 1.000 (standard error = 0.000; 95% CI 1.000-1.000, $P < 0.0001$). The perfect AUC indicates complete separation between groups, as no viability values overlapped (5 controls at 100% and 5 treated samples at around 30%). The cutoff points showed a high level of sensitivity and specificity. For example, cutoff values lower than 28.58% showed a sensitivity of 100% (95% CI 56.55%-100%) and a specificity of 100% (95% CI 56.55%-100.0%). A cutoff less than 66.67% had a perfect sensitivity of 100%. The ROC curve is shown in Figure 2 and underscores that the viability assay is an appropriate measure to detect cytotoxicity due to Azithromycin at lower concentrations.

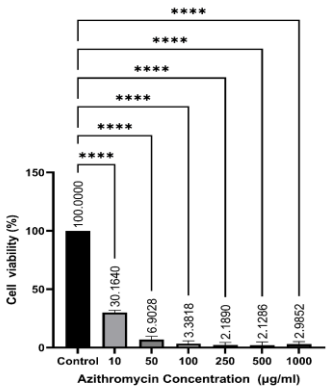

Fig. 1 Dose-Dependent Effect of Azithromycin on HT29 Cell Viability (n=5 independent experiments per group)

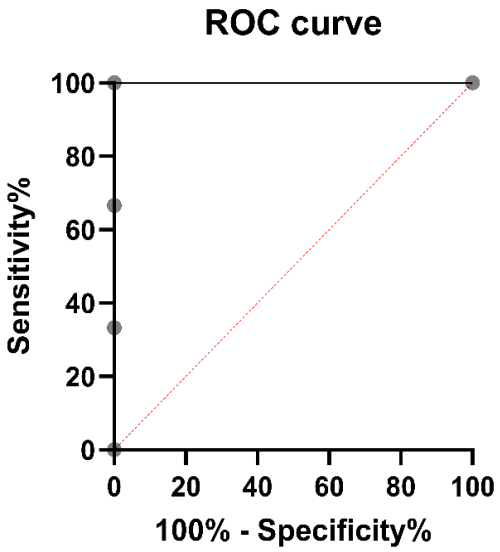

Fig. 2 Receiver Operating Characteristic (ROC) Curve for Azithromycin-Induced Cytotoxicity in HT29 Cells

Table 1 Impact of Azithromycin on HT29 Colon Cancer Cell Viability: Dose-Dependent Cytotoxicity and Statistical Analysis

Azithromycin Concentration (µg/ml)	Mean Viability (%)	Standard Deviation	Mean Difference vs. Control (%)	Adjusted P-value (Dunnett's Test)	Remarks
Control	100	-	-	-	Reference (100%)
10	30.16	3.45	69.84	<0.0001	Significant reduction
50	6.903	1.23	93.10	<0.0001	Significant reduction
100	3.382	0.67	96.62	<0.0001	Significant reduction
250	2.189	0.45	97.81	<0.0001	Significant reduction
500	2.129	0.39	97.87	<0.0001	Significant reduction
1000	2.985	0.52	97.02	<0.0001	Significant reduction

This study assessed the effects of a treatment on the relative transcript level of *Casp8* under control conditions using an unpaired t-test with Welch's correction to assess statistical significance. The data in total do not indicate a difference in *CASP8* transcript levels between treatment and control at a P-value of 0.5106 and a cutoff value at a typical significance level of 0.05. Meaning the treatment under these conditions did not have a significant effect on *CASP8* expression. The results are depicted in the bar graph "*CASP8*," which shows the mean relative transcript level of transcript expression for both groups with error bars to depict variability. The primary hypothesis assessed was whether treatment would significantly change the relative transcript level of *CASP8* as compared to the control. The mean transcript level was 1.068 for the control and 0.8491 for the treatment, for a difference of -0.2190 with a standard error of the mean (SEM) ±0.3131. The 95% CI for this difference was -0.9854 to 0.5474, which includes zero, further indicating no significant difference. The Welch-corrected t-test resulted in a t-value of 0.6993 with degrees of freedom (df) = 5.995, and the R-squared value is 0.07543, indicating a small effect size as reflected in the non-significant P-value. In an additional analysis, the assumption of equal variances across groups was tested using F-tests to compare variances. The F-test indicated an F-value of 1.062 and 3 and 3 degrees of freedom (DFn, Dfd) with a P-value = 0.9615, which is not significant ($P > 0.05$). The reported non-significant findings suggest that variances from the two groups were similar, supporting assumptions of the use of the t-test. Both the treatment and control groups had four observations; therefore, sample sizes were equivalent across groups for statistical comparisons. The bar graph depicting "*CASP8*" illustrates that the control condition has a

marginally greater mean transcript level (1.068) compared to the treatment condition mean (0.8491), though the difference was not significant ("ns") (Figure 3). Moreover, the error bars (standard deviation) do considerably overlap, lending further support that there is an observable effect. Based on these findings, further experimentation with increased sample sizes or different treatment conditions will be needed to observe subtle effects on the expression of *CASP8*.

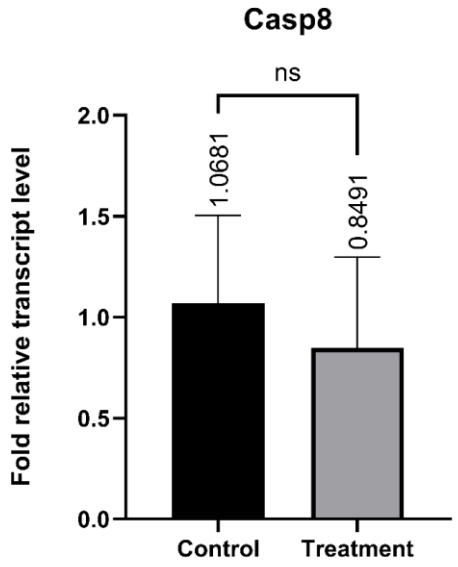

Fig. 3 Comparative Analysis of *CASP8* Transcript Levels Between Control and Azithromycin-Treated Groups (n=4 per group)

Discussion

Azithromycin's marked cytotoxicity in HT29 colon cancer cells, summed up by a dose-related viability decrease that stabilizes at higher concentrations, supports the promise of Azithromycin as an antiproliferative agent in colorectal cancer models. The evidence seems to cohere with the hypothesis through which this research - to determine if Azithromycin inhibits cancer cell growth would have predicted this viability decrease for the colon cancer cells from a control of 100% viability to as low as 2-3% at doses above 100 µg/ml, and this prediction was statistically validated using ANOVA and ROC analysis (Toriyama et al., 2021; Yamamoto et al., 2018). Furthermore, no significant change in *Casp8* transcript levels was observed, suggesting that Azithromycin's cytotoxicity may not primarily involve the extrinsic apoptotic pathway at the transcriptional level; however, mRNA data alone are insufficient to exclude caspase involvement, and further assays (e.g., Western blot for caspase-8 protein and cleavage, caspase activity assays, PARP cleavage, annexin V/PI flow cytometry for apoptosis, LC3-I/II and p62 for autophagy, and lysosomal membrane permeabilization assays) are required to support mechanistic claims. Overall, these speculations offer the notion that Azithromycin offers a unique role as treatment that could potentially operate in a multifactorial manner, further suggesting a potential role as a targeted cancer therapy and not just antibiotic treatment. The observed dose-dependent cytotoxicity in the present study aligns with other studies, which have reported Azithromycin anticancer mechanism in colon cancer models, significantly in the context of modulation of cellular processes such as autophagy. For example, it was reported that Azithromycin enhanced the activity of TRAIL in colon cancer cells through inhibition of autophagy and upregulation of death receptors. This also supports our substantial reductions in viability despite combination therapies (Qiao et al., 2018; Xu et al., 2023). In addition, studies using macrolides such as clarithromycin, a structural analog of Azithromycin, have also found inhibition of colorectal cancer growth through autophagic flux inhibition, which extends our understanding that Azithromycin may act similarly in HT29 cells (Petroni et al., 2020; Yu et al., 2023). This congruence supports a conserved mechanism present in macrolides and further supports Azithromycin's potential repurposed drug for improving the efficacy of chemotherapy (Kudo et al., 2023; Xu et al., 2023). Nonetheless, the plateau of cytotoxicity at higher doses is where the authors' findings depart from some of the literature that has focused on either dose-dependent cytotoxicity or dose-responses (Kudo et al., 2023). Research has indicated synergistic anti-proliferative effects of Azithromycin in combination with vincristine, with no indication of saturation of cytotoxicity in cervical and gastric cancer (Sun et al., 2012; Zhang et al., 2012). This divergence could suggest that an HT29-specific resistance mechanism, specifically the action of one of those efflux pumps or adaptive stress responses to the azithromycin, has inhibited it from achieving optimal efficacy at high concentrations while implying potentially even more complicated

strain-dependent pharmacodynamics therein (Pasipanodya et al., 2012). The perfect ROC AUC for the low-dose component of Azithromycin may further contextualize this work, but should be validated across more replicates and cell lines before claiming diagnostic utility (Zhao et al., 2024).

The absence of significant change in the expression of Casp8 diverges from the literature that associates Azithromycin with apoptosis, likely occurring via mitochondrial or lysosomal pathway, and not through the death receptor pathway (Toriyama et al., 2023; Toriyama et al., 2018; Kudo et al., 2023; Xu et al., 2023). For instance, Azithromycin increases the cytotoxicity of DNA-damaging drugs by promoting lysosomal membrane permeabilization in lung cancer and does so in a way that does not involve *CASP8*, supporting our interpretation of alternative apoptotic pathways in our HT29 cells (Toriyama et al., 2023). This is an extension of literature stating that, while there are some pro-apoptotic effects covered in mini reviews for Azithromycin, without modulation of *Casp8* here, likely due to the context being important with regulation in hypoxic or normoxic conditions (Mohamed et al., 2023; Yu et al., 2023). These differences reinforce that pathway-specific considerations are crucial to reconcile these observations (Zhou et al., 2022). While these discoveries serve to advance the knowledge of the metabolic and cell death pathway that underpins the tumor and TME cell biology, the investigator recognize limitations with respect to only being able to investigate a single cell line (possibly limiting generalizability to the heterogeneous nature of colorectal tumors) and also having small sample sizes for the analysis of *CASP8* (n=4 per group), which might lead to decreased power for small effect sizes. The studies are performed in vitro and therefore ignore important pharmacokinetic variables such as bioavailability in vivo. Future studies should assess multiple cell lines, undertake pre-clinical animal studies (using these lines), and explore combinatorial treatment options in which an autophagy inhibitor is used in conjunction with another chemotherapy agent to further characterize the mechanism of *CASP8* action in colorectal cancer. Proteomic studies could also further elucidate the post-transcriptional role of *CASP8* in therapeutic response and could inform attack design with a more specific mechanism of action.

Conclusion

In conclusion, this study identified a substantial dose-dependent cytotoxic effect of Azithromycin on the HT29 colon cancer cell line, with viability assaying demonstrating viability from 100% in controls to about 2-3% of cells viable in concentrations of 100 μg/ml or greater, through viability assays and confirmed through one-way ANOVA ($F(6,28) = 120.5$, $P < 0.0001$) and ROC curves (AUC = 1.000). These results confirm the primary hypothesis of Azithromycin inhibiting HT29 cell proliferation, with evidence of a plateau in effect at higher doses noted, which indicates that the antiproliferative effects may be saturated. Controlling for apoptosis through assessing the levels of *Casp8* transcripts revealed no significant differences between control and treated groups ($P = 0.5106$), rejecting the secondary hypothesis of *Casp8* involvement with Azithromycin treatment at the transcriptional level and further clarifying that Azithromycin treatment effects may be mediated through alternative pathways. The wider implications of these findings highlight Azithromycin as a potential repurposed therapeutic agent in colorectal cancer treatment by enhancing chemotherapy by virtue of its antiproliferative effects, thereby improving the prognosis in chemotherapy-resistant models of cancer. The study identifies no observed *Casp8*-dependent mechanisms at the mRNA level, raising the question of whether Azithromycin might have clinical utility in precision oncology with targeted applications, including the potential for combinations with autophagy-inhibiting agents. Given these implications, further investigation into Azithromycin as a potential repurposed drug will require the implementation of in vivo studies to specifically evaluate the translatability of the findings *in vivo* protocols, as well as general molecular pathways, improved visibility into other cellular pathways impacted by Azithromycin (for instance: autophagy). Finally, outcomes obtained here point to the potential for investigational drug combinations with anticancer agents currently in use, but additional mechanistic experiments (caspase activity, apoptosis/autophagy markers) are needed before translational claims.

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