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Experimental and computational determination of synergy between ascorbic acid and ivermectin against cervical cancer HeLa cells

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ABSTRACT

Cervical cancer remains a global health burden disproportionately affecting low- and middle-income countries (LMICs) due to inaccessible prevention strategies, screening, and treatment. In this exploratory, in vitro proof-of-concept study, we investigated the potential synergistic antiproliferative effect of combining ivermectin and ascorbic acid against the cervical cancer model (HeLa) cell line. Antiproliferative effects of ascorbic acid and ivermectin were evaluated in HeLa cell lines using the MTT assay. Using experimentally calculated half maximal inhibitory concentration (IC_{50}) values for ivermectin and ascorbic acid, the two drugs were combined, and their interactions were analyzed using Compusyn software. Computational prediction was performed using network pharmacology and molecular docking studies. Gene expression analysis was also performed to provide functional validation of putative targets by assessing their deregulation at the transcriptional level. IC_{50} values of ivermectin and ascorbic acid on HeLa cells were 9.65 µM and 4.58 mM, respectively. Analysis using Compusyn software for the interaction between ascorbic acid and ivermectin showed an additive effect near the IC_{50} and synergy at higher concentrations (>IC_{50}). Network pharmacology and molecular docking predicted putative genes and pathways for the combination treatment. Further, gene expression analysis on selected genes showed upregulation of *TP53* and downregulation of *STAT3*, *BCL2*, *HIF1α*, and *TNF*. Overall, this exploratory proof-of-concept study demonstrates dose-dependent antiproliferative activity between ivermectin and ascorbic acid, though with weak synergism.

INTRODUCTION

Cervical cancer is the fourth most common type of malignancy and the leading cause of cancer-related mortality among women globally [1]. This malignancy is mainly caused by infection with the human papillomavirus (HPV), specifically the high-risk strains 16 and 18, which result in the expression of the E6 and E7 proteins, which are responsible for carcinogenesis [2,3]. The burden of the disease is borne heavily by low- and middle-income countries (LMICs) [4]. For instance, in Sub-Saharan Africa, more than 85% cases are registered with higher mortality rates of up to 87% due to cervical cancer [5]. The survival rates following cervical cancer diagnosis in this region are as low as 35% [6]. The '90-70-90' World Health Organization (WHO) provides a great framework for controlling this malignancy [7]. However, implementing this initiative remains a challenge for countries with limited resources [4]. There is a need, therefore, to come up with alternative strategies to address cervical cancer inequalities.

Treatment approaches for cervical cancer involve surgery, radiotherapy, chemotherapy, and immunotherapy [6]. However, treatment disparities are evident in resource-limited regions where patients lack advanced cancer therapies [8]. Conventional chemotherapy often includes treatment using cisplatin, a platinum-based drug, which is associated with various side effects such as bone marrow suppression, nephropathy, ototoxicity, vomiting, and nausea [9,10]. Unfortunately, few drugs are being developed targeting cervical cancer, considering the high cost of drug discovery. Use of natural products and their combinations for cervical cancer treatment has also been suggested as an alternative approach for cervical cancer treatment [11,12]. A "low-hanging fruit" to bridge the gap is undertaking drug repurposing, which has gained attention in oncological settings due to reduced drug development period and known safety profiles of drugs [13,14]. Several drugs have been studied for repurposing in cancer treatment, including artemisinin, ivermectin, ascorbic acid, vitamin D, zinc, mebendazole, quercetin, and metformin [15]. They exert antitumor effects through various mechanisms, including suppressing invasion and metastasis, decreasing angiogenesis, blocking phenotypic plasticity, controlling the tumor microenvironment, targeting senescent cells, targeting cancer stem cells (CSCs), and mitochondrial-mediated effects [14,15]. Presently, one of the effective cancer treatment strategies involves a combination of drugs that mechanistically target the bulk of tumor cells and CSCs [15]. Moreover, agents that target cancer metabolism and CSCs have been proposed as another rational combination approach for cancer treatment [16]. Using the drug repurposing approach through the combination of ivermectin and ascorbic acid is particularly relevant in LMICs since the two drugs are inexpensive and widely accessible.

Ivermectin, an antiparasitic drug, is reported to induce apoptosis, cell cycle arrest, and cause mitochondrial damage in cancer cells [17,18]. Studies have shown that ivermectin can regulate glucose metabolism in cancer, reversing the Warburg effect experienced in cervical cancer [19]. Ascorbic acid, on the other hand, has been reported to have a pro-oxidant effect at pharmacological concentrations that cause selective cytotoxicity against cancer cells, with suggested bioactivity against CSCs [15,20]. Studies have further demonstrated that ascorbic acid induces metabolic changes by altering the concentration of reactive oxygen species (ROS), which is critical for targeting CSC-like populations [21,22]. Phase 1 clinical trials of ivermectin and ascorbic acid for cancer treatment have reported the safety of the two drugs for human use [23–25]. Studies have recommended a combination treatment of micro-elements and off-label drugs as potential agents in targeting cancer and CSCs [15,26]. The rationale for the combination is that ivermectin has been implicated in inducing cell death through the mitochondrial pathway, while ascorbic acid is highly potent in targeting CSCs [26]. Therefore, a combination of these attributes is a desirable strategy in cancer therapy at the moment [15,16].

This exploratory work sought to test this strategy by investigating the possible combination and interaction between ivermectin and ascorbic acid against cervical cancer model HeLa cells. To the best of our knowledge, synergy between ivermectin and ascorbic acid in cervical cancer model HeLa cells has not been reported before. This *in vitro* study, therefore, used experimental cellular and molecular methods to determine the antiproliferative potential of the combined treatment of ivermectin and ascorbic acid, and their possible synergism. Network pharmacology, molecular docking, and gene expression analysis were integrated to provide a mechanistic understanding of the demonstrated bioactivity [27].

MATERIALS AND METHODS

Cell culture

HeLa cells (HeLa-229 (ATCC CCL-2.1)) were obtained from the American Type Culture Collection (ATCC, USA) and were maintained in EMEM Media supplemented 10% Fetal Bovine Serum (FBS, Gibco, USA), 1% of a 1M HEPES buffer solution (Sigma Aldrich, USA), 1% Penicillin/Streptomycin (Gibco, USA), 7.5% Sodium Hydrogen Carbonate (Sigma, USA), 1% L-Glutamine (Sigma, USA), and 1.25 mg/L Amphotericin B (Sigma, USA). The cells were cultured in a humidified incubator (Thermo Fisher Scientific, USA), maintained at 5% CO_2 and 37°C. When the cells achieve a confluence level of over 80% and enter the exponential growth phase, they are regarded as confluent and sub-cultured.

Antiproliferative assay

The cells were treated at different concentrations of ivermectin (Zenex Animal Health Pvt Ltd, India) and L-ascorbic acid (Loba Chemie Pvt Ltd, India). 40 μM ivermectin and 40 mM ascorbic acid were prepared 0.2% DMSO. L-Ascorbic acid solutions were prepared fresh and used immediately to avoid degradation. HeLa cells, seeded at 10,000 cells/well in 96-well plates in EMEM with 10% FBS, were treated with serial dilutions of ivermectin (0.6, 1.25, 2.5, 5, 10, 20, and 40 μM) and ascorbic acid (0.6, 1.25, 2.5, 5, 10, 20, and 40 mM) for 48 hours. The positive control was cisplatin (United Biotech Pvt Ltd, India). Cell viability was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent (Solarbio, China), and the formazan crystals were dissolved using DMSO, and the absorbances were read at 570nm and 720nm using a plate reader (Infinite M1000 by Tecan) [28]. The reaction of ascorbic acid with MTT has been reported before; therefore, the wells were washed twice with PBS [29]. Cell viability was calculated according to the following equation as described previously [28]:

$$Cell\ viability = \frac{Absorbance\ of treated\ cell - Absorbance\ of culture\ medium}{Absorbance\ of untreated\ cell - Absorbance\ of culture medium} \times 100$$

Combination treatment

The IC_{50} results for both ivermectin and ascorbic acid were used to perform the combination. The doses were used to perform the drug combinations using the Latin square diagonal method described by Chou and Talalay, as shown in (Supplementary Table 1). The doses of ivermectin and ascorbic acid were mixed in a 1:1 ratio based on the IC_{50} concentrations [30]. The CompuSyn software was used for drug analysis to identify drug interaction parameters, including dose-effect analysis, dose-response curves, and the determination of the Combination Index (CI). Synergism is often indicated by a CI value less than 1, antagonism by a CI value greater than 1, and an additive effect by a CI value of 1 for a medication combination [31]. The positive control was cisplatin, a conventional drug for cervical cancer treatment, and the negative control was cells with no treatment and cells treated with 0.2% DMSO (used to dissolve the drugs). Cell viability was assessed using the MTT assay as described by [32].

Cell morphology analysis

The effects of ivermectin, ascorbic acid, and their combination on morphological changes in HeLa cells were evaluated under the EVOSTM XL (Thermo Fisher Scientific, USA) digital microscope at ×20 magnification to observe changes in the cells [28].

Wound healing assay

HeLa cells were plated in 6-well plates at 2.5 × 10^5 cells/mL, and a sterile 200 µl pipette tip was used to scratch a line on an 80% confluent plate. The plates were washed twice with PBS and then subjected to ascorbic acid, ivermectin, or their combination. Images were taken using the EVOSTM XL (Thermo Fisher Scientific, USA) microscope after an hour, 24 hours, and 48 hours of treatment. Wound width was analyzed by the ImageJ software [12]. The rate of migration was calculated using the following equation as described previously [28]:

$$Relative\ migration\ index(\%) = \frac{scratch\ distance\ at\ 0hr - scratch\ distance\ at\ time\ (hr)}{scratch\ distance\ at\ ohr} \times 100$$

Clonogenic assay

The colony formation (clonogenic) assay has been used to measure the capacity of CSCs to proliferate and form colonies [33]. We used it to determine the potency of ivermectin and high-dose ascorbic acid in targeting CSCs. HeLa cells were seeded at a density of 500 cells/mL in a 6-well plate. The clonogenic assay was then performed as described by [34] with slight modifications. Briefly following seeding and overnight incubation at 5% CO_2 and humidity, the cells were treated with a predetermined IC_{50} concentration of ivermectin, ascorbic acid, and the combination of the two based on Compusyn software analysis. The concentrations were then diluted as IC_{50}, IC_{25}, $IC_{12.5}$, and $IC_{6.25}$, with untreated and cisplatin serving as negative and positive control, respectively. The cells were incubated at 5% CO_2 and humidity for 24 hours, after which the treatment media was removed, and new media was added, and continuous replenishment was undertaken every 3 days for 14 days. After this, the colonies were washed gently using PBS and fixed using 1 mL 3.7% formaldehyde in each well overnight at room temperature. This was followed by the addition of 1mL 0.05% crystal violet solution and subsequent incubation at room temperature for 2 hours [35]. The plates were then washed with water and left to air dry. Colonies with more than 50 cells were counted using the microscope EVOSTM XL (Thermo Fisher Scientific, USA).

DNA fragmentation assay

To determine apoptosis, a DNA fragmentation assay was performed [34]. HeLa cells were introduced in T25 flasks at a density of 10^5cells/mL and incubated overnight at 37^0C °C and 5% CO_2. They were then subjected to a combination of ivermectin and ascorbic acid at IC_{75} and IC_{50} or ivermectin (5µM) or ascorbic acid (2.5mM) alone, alongside a negative control of untreated cells. These treatments proceeded for 48 hours, and the cells were collected using trypsinization, centrifuged, and the pellet was resuspended in 100µL PBS for DNA extraction as explained by [34] with a few modifications. DNA was extracted using the DNeasy® Blood & Tissue Kit following the manufacturer's protocol. The protocol for gel electrophoresis was done as explained by Lee et al (2012) [36].

Targeting cervical cancer genes with l-ascorbic acid and ivermectin

The Simplified Molecular Input Line Entry System (SMILES) format of L-ascorbic acid and ivermectin were obtained from Pubchem and CHEMBL, respectively (accessed on 20 May 2025). To predict the molecular targets of these compounds, we used SwissTargetPrediction, Super-PRED, Way2Drug, and Similarity Ensemble Approach (SEA) with "homosapiens" as the species of study (all accessed on 20 May 2025). Cervical cancer genes were obtained from GeneCards, PHAROS, NCBI, and Open Targets platforms with "Cervical cancer" as the disease search term (all accessed on 20 May 2025) [12]. The prediction results from the databases were pooled together after homogenizing them using the Uniprot Mapping tool (accessed on 21 May 2025), and the cervical cancer genes from various databases will also be combined. Redundant genes were identified from each set of data and removed. We intersected the drug targets and cervical cancer (cacx) genes to form a Venn diagram using the Bioinformatics and evolutionary genomic tools (accessed on 21 May 2025), to find common gene targets and outstanding genes [28].

Protein-protein interaction network analysis

The protein-protein interaction (PPI) network was established by uploading the ivermectin and ascorbic acid targets and some shared targets with gene databases to the STRING platform (accessed on 22 May 2025) and selecting "Homo sapiens" in the organism search field [37]. In the Basic Setting and Advanced Setting columns, the options "Medium Confidence (0.400)" and "Hide disconnected nodes in the network" were selected, respectively. The output was generated in the form of Tab-Separated Values (TSV) format, and the PPI network was analysed using the Cytoscape 3.10.3 application for hub gene identification. The top 30 hub genes were selected for molecular docking [28]. The genes were analysed based on degree, Maximum Neighborhood Component (MNC), closeness centrality, and Maximal Clique Centrality (MCC), and common genes were subjected to molecular docking [38].

GO and KEGG enrichment pathway analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed via the online enrichment tool ShinyGO version 0.77, and parameters will be set as: species "human " and the false discovery rate (FDR) will be 0.05 [12]. Enriched GO terms were assessed based on three categories: Biological Process, Molecular Function, and Cellular Component [38]. Further KEGG pathway analysis was performed using the SRplot tool.

Molecular docking analysis

The Structure Data Format (SDF) format for ivermectin (DB00602) used in molecular docking was obtained from Drug Bank, and ascorbic acid (54670067) was obtained in SDF format from Pubchem. These ligands were prepared for docking using the Open Babel tool embedded in PyRx software. The ligands were minimized in a total of 200 steps and converted to AutoDock compatible 'pdbqt' format for docking [28]. The top 30 hub genes were subjected to molecular docking. The structures for the protein target receptors were obtained from the Protein Data Bank (PDB) in PDB format. UCSF ChimeraX 1.10 was used to prepare the proteins by removing non-standard residues, water molecules, heteroatoms, non-target chains, and ligands. Structure editing on the

protein was performed using Dockprep to add hydrogen bonds and Gasteiger charges. Target residues for the binding pocket were retrieved from previous reports. Docking was carried out using the Vina tool built in the PyRx Program, and docking was validated using the native ligands co-crystalized with proteins. The grid box was centralized based on active amino acid residues of the binding pocket of each protein, which were highlighted by a feature in PyRx known as "Toggle Selection Spheres," and the docking was set at the exhaustiveness of 8 [39]. The docking poses with the lowest binding affinities and null root mean square deviation (RMSD) were selected for analysis using the Discovery Studio 2025 Client tool (BIOVIA) [28].

Gene expression analysis

This study was performed to determine the relative expression of tumor protein p53 (*TP53*), signal transducer and activator of transcription 3 (*STAT3*), and B-cell lymphoma 2 (*BCL2*), hypoxia-inducible factor 1 alpha (*HIF1α*), and tumor necrosis factor (*TNF*). Total RNA was first extracted from cells treated with IC_{50} concentrations of the combination, ascorbic acid, or ivermectin and a negative control (untreated cells) using Quick-RNA™ Miniprep Kit (Zymo Research) following the manufacturer's protocol. The quality and quantity of the RNA were measured using the Jenway Genova nanodrop machine. Following RNA extraction, cDNA was synthesized using the LunaScript® RT SuperMix Kit (New England Biolabs, Germany) following the manufacturer's protocol. The quantitative real-time polymerase chain reaction (qPCR) was performed using Luna® Universal qPCR Master Mix (New England Biolabs, Germany) based on the manufacturer's protocol. The program for running the qPCR was set as follows: initial denaturation at 95°C for 60 seconds, denaturation at 95 °C for 15 seconds, extension 60°C for 30 seconds (40 cycles), and the melting curve at 80-85°C. *GAPDH* and β-actin were used as the reference gene, and the relative expression was calculated using the $2^{-\Delta\Delta ct}$ method [12]. The sequences of the primers used are shown in Table 1.

Table 1. Primers used for gene expression studies.

Genes	Sequences
STAT3	F: 5′-AAACAGCAGGATGGCCCAAT-3′ R: 5′-ACATCCTGAAGGTGCTGCTC-3′
Bcl-2	F: 5′-GAACTGGGGGAGGATTGTGG-3′ R: 5-CATCCCAGCCTCCGTTATCC-3′
TP53	F: 5′ TGTGGAGCAGGAAAGAAGTGGC -3′ R: 5′- AGGGAGTTGGGAATAGGGTG-3′
HIF1a	F: 5′-TCAAAGTCGGACAGCCTCAC-3′ R: 5′-TAGCTGCATGATCGTCTGGC-3′
TNF	F:5′-ACTACCAGCAATCCCCATCT-3′ R: 5′-AGAGTTCAGTGATGTAGCG-3′
β-actin	F: 5′- CTTCCAGCCTTCCTTCCTGG-3′ R: 5′- CTTCTGCATCCTGTCGGCAA-3′
GAPDH	F: 5′-GCTCCCACCTTTCTCATCCA-3′ R:5′-TACTCCCCACATCACCCCTCTA-3′

Statistical analysis

Statistical data analysis was performed using GraphPad Prism 8.0 (San Diego, California, USA) to compare differences in means using the One-way ANOVA and Tukey and Dunnett's post hoc test. Statistically significant differences were relative to the negative control (cells treated with 0.2% DMSO), denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. The data is presented as the mean ± SD.

RESULTS

Antiproliferative activity and combination analysis of ivermectin and ascorbic acid on HeLa cells

The antiproliferative activity of ivermectin and ascorbic acid on HeLa cells was investigated in a double-dilution assay to determine the half-maximal inhibitory concentration (IC_{50}) of each drug using the MTT assay. Both ivermectin and ascorbic acid inhibited the proliferation of HeLa cells in a dose-dependent manner (Figure 1 A & B). Ivermectin and ascorbic acid showed an IC_{50} of 9.64 µM and 4.58 mM, respectively, as calculated from data shown in (Figure 1 A and B).

HeLa cells were then subjected to combination treatment (ivermectin and ascorbic acid) or to individual treatments. As shown in Figure 1C, the combination had a more significant antiproliferative effect than individual treatments ($p<0.0003$). To analyze the interactions between the two compounds, we used the Compusyn software. The results show that the two compounds exhibited synergistic interactions at IC_{75} and IC_{90}, with an additive effect near IC_{50}, as depicted in Figure 1D. The results of the dose reduction index are presented in Supplementary Figure 1. Table 2 was constructed from the contents of the Compusyn report showing the parameters and fraction affected (fa) for each compound and the combination with their corresponding synergy score. Interestingly, at higher fa values, there is improved synergy.

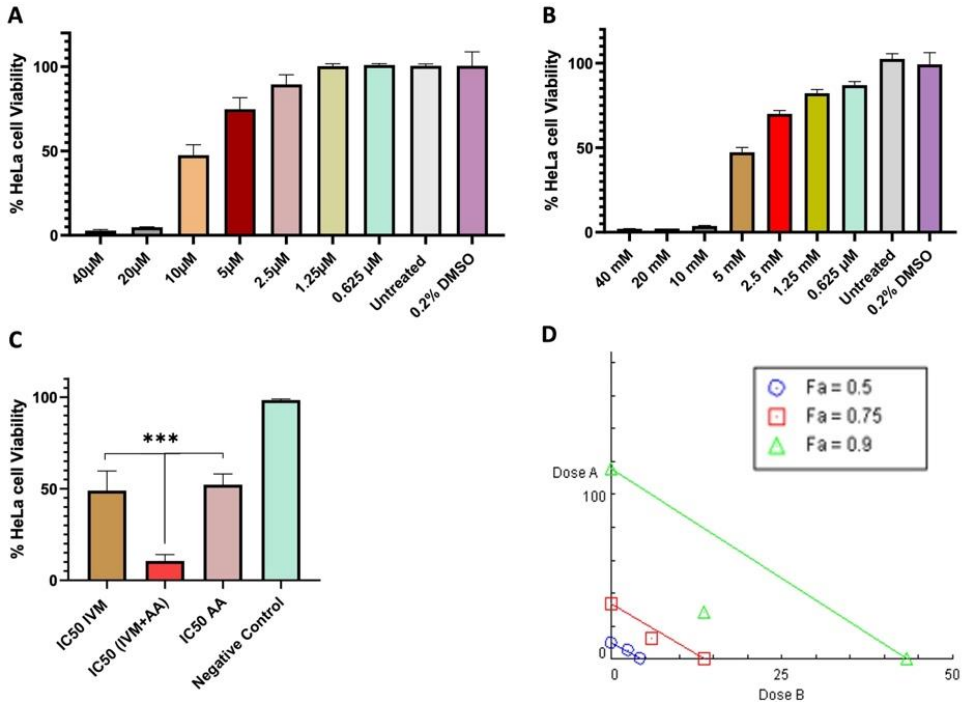

Figure 1. Antiproliferative activity testing. Dose-dependent antiproliferative activity of A) Ivermectin and B) Ascorbic acid. All treatments were performed in technical triplicates (n=3) and repeated 4 times (biological repeats). C) Percentage proliferation at IC_{50} between combination and individual treatments. Negative control cells were exposed to media containing 0.2% DMSO. D) The isobologram analysis for synergy determination showing additive effect at fa 0.5 and synergy at higher doses (fa 0.75 and fa 0.9). The diagonal line shows the threshold for antagonism, additivity, or synergism at the given concentration (fraction affected, fa values). IVM: Ivermectin; AA: ascorbic acid; IC_{50}: inhibitory concentration that killed 50% of cells; Fa: fraction affected. Statistical significance is denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Table 2. Synergistic interactions of ivermectin and ascorbic acid.

Compound		Parameters				
Ivermectin	**Ascorbic Acid**	**fa**	**m**	**dm**	**R**	**CI**
38 µM		0.96	0.889+/-0.17	9.74	0.92	
19 µM		0.92				
9.5 µM		0.5				
4.75 µM		0.18				
2.4 µM		0.04				
1.2 µM		0.02				
0.001 µM		0.001				
	18 mM	0.95	0.95 +/- 0.16	4.2	0.94	
	9 mM	0.92				
	4.5 mM	0.49				
	2.4 mM	0.21				
	1.2 mM	0.07				
	0.6 mM	0.02				
	0.001 mM	0.001				
38 µM	18 mM	0.97	1.31+/- 0.19	7.89	0.95	0.37546
19 µM	9 mM	0.94				0.44482
9.5 µM	4.5 mM	0.84				0.65506
4.75 µM	2.3 mM	0.34				1.39833
2.4 µM	1.2 mM	0.06				2.97861
1.2 µM	0.6 mM	0.02				2.97861
0.002 µM	0.001mM	0.001				2.97861

Synergy analysis from the Compusyn report showing the fraction affected (fa), coefficient signifying dose effect relationship (m), median effect dose-IC50 (dm), correlation coefficient (R), and the combination index (CI). CI values <1 show synergy, =1 show additivity, and >1 show antagonism.

Effect of ivermectin and ascorbic acid on the morphology of HeLa cells

From the Compusyn analysis report, concentrations at IC_{50} and IC_{75} were obtained. The cells were observed for morphological changes at IC_{75}, IC_{50}, and compared with individual treatments at their IC_{50} concentrations, as shown in Figure 2. These results show that the positive control and the treatments exhibited cell death, detachment, and cellular shrinkage. Further, at the combination level, more morphological changes are observed than with ascorbate alone, but similar to those with ivermectin.

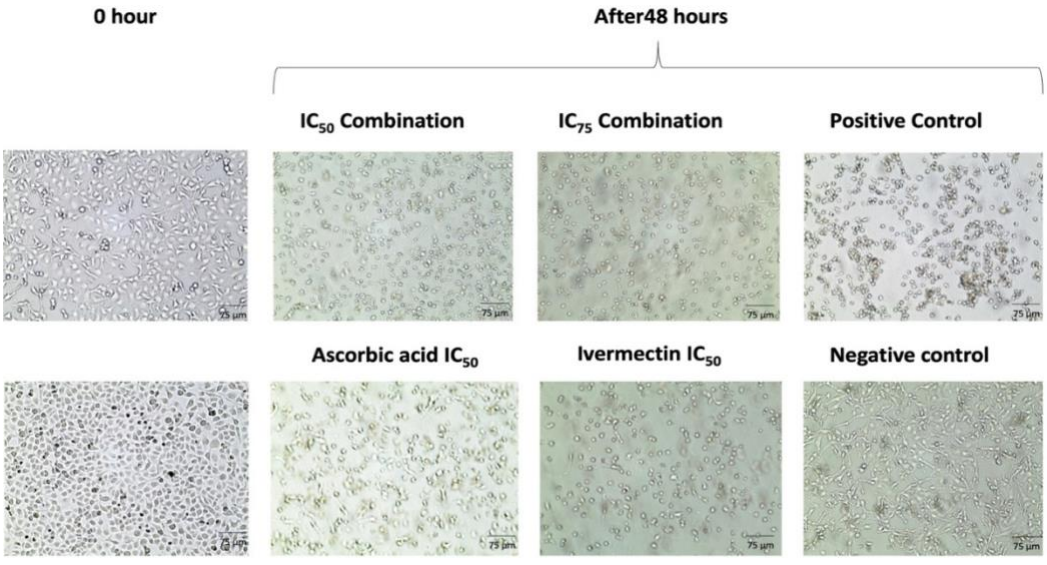

Figure 2. Cell morphology changes after exposure to a combination of ivermectin and ascorbic acid at IC_{50} (5.0 µM ivermectin, 2.5mM ascorbic acid, positive control cisplatin solution (40µM)), and negative control.

Effect of ivermectin and ascorbic acid on wound healing in HeLa cells

We further interrogated the effect of the combination treatment versus individual compounds on wound healing. Interestingly, both the combination and the individual compounds significantly inhibited wound healing compared with untreated cells ($p<0.0001$ for all treatments), as shown in Figure 3. There were no significant differences between the combination and individual treatments in wound closure ($p>0.999$).

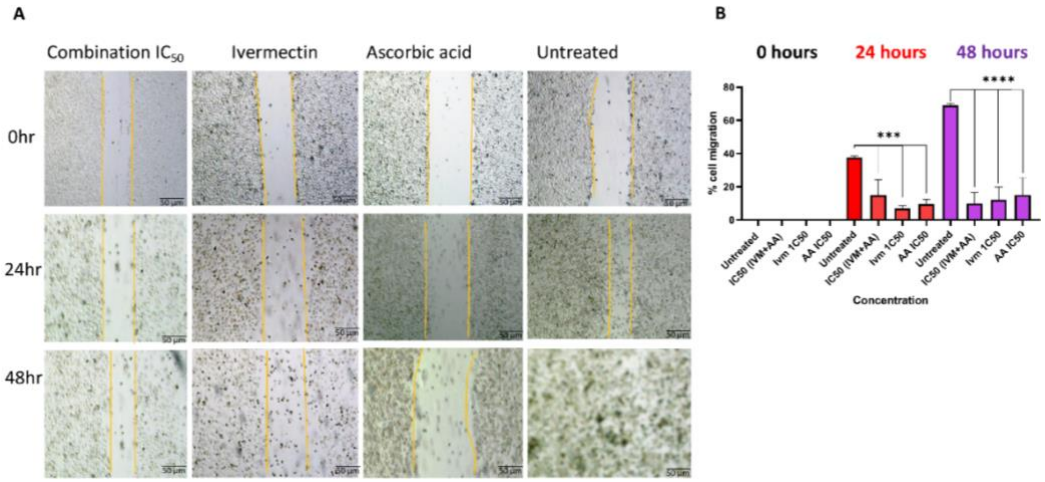

Figure 3. Effect of ivermectin and ascorbic acid on wound healing. A) shows the wound-width following treatment using the combination treatment at IC_{50} versus ascorbic acid and ivermectin alone at 0 hours, 24 hours, and 48 hours. B) shows a graph of relative cell migration following treatment using either combination, ivermectin or ascorbic acid. Three technical and three independent experiments were performed for the mean and SD values. IC50 (IVM+AA): Combination treatment; IVM: Ivermectin; AA: Ascorbic acid. Statistical significance is denoted by $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

Effect of ivermectin and ascorbic acid on colony formation in HeLa cells

This assay was performed to determine the activity of the treatments against clonogenic potential and stemness of single cells by analyzing the ability of a single cell to form colonies. Our results show that the combination treatment and ascorbic acid treatment eradicated all colonies compared to the untreated. There were significant differences in the ability to prevent colony formation at $IC_{12.5}$ with ivermectin compared to IC_{50} and IC_{25} ($p<0.0001$). Interestingly, there were no differences in the colony eradication in the combination treatment and ascorbic acid, as shown in Figure 4.

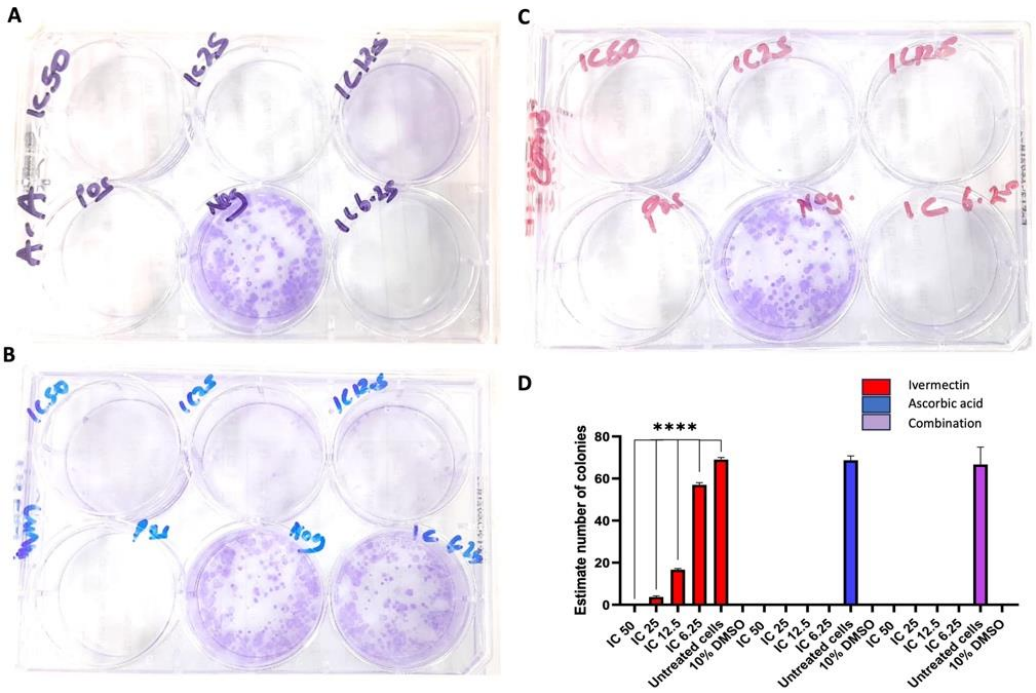

Figure 4. Colony formation. After treatment with A) ascorbic acid, B) ivermectin, and C) a combination of the two at IC_{50}, IC_{25}, $IC_{12.5}$, $IC_{6.25}$, Negative control, and positive control, respectively. D) The bar graph shows a quantitative estimation of the number of colonies per treatment. Three independent experiments were performed for the mean and SD values. Statistical significance is denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Effect of ivermectin and ascorbic acid on DNA fragmentation in HeLa cells

Based on our morphological studies, we hypothesized that our treatment could be killing HeLa cells through the induction of apoptosis. To test this hypothesis, a DNA fragmentation assay was performed. Interestingly, DNA fragmentation was observed in IC_{75} and IC_{50} treatments of the combination, more than in individual treatments with ascorbic acid and ivermectin, as shown in Figure 5.

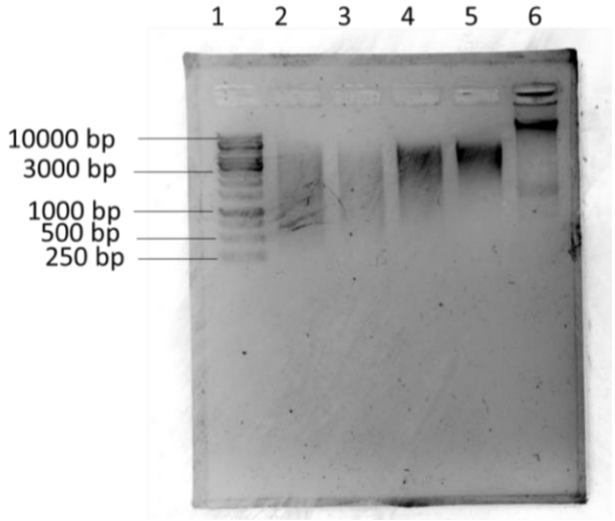

Figure 5. DNA fragmentation assay representing DNA extracted from cells treated with either combination treatment, ivermectin, or ascorbic acid. (lanes 1-6) 1- DNA ladder, 2-IC_{75} combination treatment, 3-IC_{50} combination treatment, 4-5µM ivermectin, 5- 2.5 mM ascorbic acid, and 6- untreated cells (negative control). DNA smearing represents DNA damage, while an intact band illustrates intact DNA.

Target prediction for ivermectin and ascorbic acid, and cervical cancer

The targets for ivermectin and ascorbic acid were predicted using SwissTargetPrediction, Super-PRED, Way2Drug, and SEA. The gene lists from the four databases were compiled to have a total of 210 targets for Ascorbic acid and 260 targets for ivermectin, forming a pool of 470 potential targets. Cervical cancer-associated genes were retrieved from GeneCards (6560), NCBI (1440), open targets platform (6502), and PHAROS (259). We compiled the cervical cancer genes in a single column, and duplicates were removed, leaving 9224 unique cervical genes. Common genes between cervical cancer and predicted targets of our compounds were identified using a Venn diagram. The results show that 92 are unique targets shared for Ascorbic acid and cervical cancer, 133 are unique targets shared for Ascorbic acid and cervical cancer, and 84 are common shared target genes among ascorbic acid, ivermectin, and cervical cancer. The Venn diagram in Supplementary Figure 2 shows the common genes between the compounds and cervical cancer genes.

PPI network construction and identification of top hub genes

From the 309 common genes, a protein network of 308 nodes and 4037 edges was generated with an estimated average node degree of 26.2 and an average local clustering coefficient of 0.47. The findings that this protein-protein interaction had significantly more interactions than expected, with a PPI value of < 1.0e-16, indicate that there are more interconnections among the constituent genes.

Further analysis using the Cytoscape application (version 3.10.3) of the network identified 30 top hub genes based on degree, Maximum Neighborhood Component (MNC), closeness centrality, and Maximal Clique Centrality (MCC) as represented in Figure 6. The 30 top genes from each calculation model were intersected to obtain core genes, as shown in the Venn diagram in Figure 6C. 24 hub genes (Figure 6C) were obtained from the intersection of the four metrices used in the calculation. These include *MMP2, HSP90AB1, MDM2, TLR4, MTOR, NFKB1, JAK2, PPARG, GSK3B, IL1B, STAT3, MAPK1, JUN, BCL2, MCL1, BCL2L1, HSP90AA1, HIF1A, RELA, PRKACA, TP53, MMP9, PTGS2, and TNF*. The results for each scoring matrix after Cytoscape analysis are presented in Supplementary Table 2.

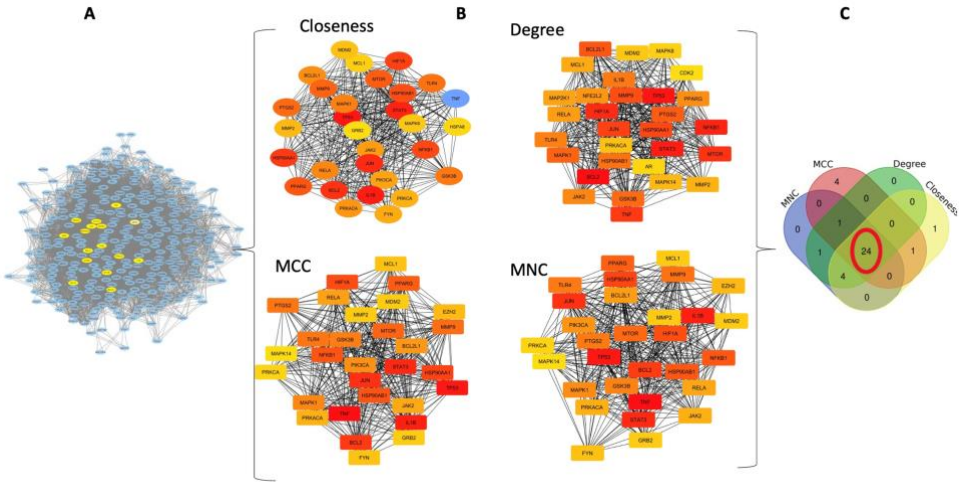

Figure 6. Network of ivermectin and Ascorbic acid with cervical cancer genes, with some top genes highlighted. A) shows the protein-protein interaction network of the genes, and B) shows different scoring parameters to obtain the top 30 hub genes. C) Venn diagram of the top hub genes following analysis by the cytohubba plugin in Cytoscape. (Maximum Neighborhood Component-MNC, Maximal Clique Centrality-MCC).

GO and KEGG enrichment pathway

GO and KEGG analyses were performed using the ShinyGO 0.82 server (species: human; $p<0.05$) to explore anti-cervical cancer-associated pathways of ivermectin- and ascorbic acid-target genes. Our findings show that the 309 targets of the combination of ivermectin and ascorbic acid were significantly enriched in 236 pathways ($p<0.05$), suggesting that these compounds act on multiple genes, targets, and pathways.

A total of 2112 GO terms were retrieved, with 1000 terms under biological processes (BP), 359 GO terms under cellular component (CC), and 753 terms under molecular functions (MF). The top enriched biological processes were response to chemicals, response to organic substances, cellular response to oxygen-containing compounds, response to chemical stimulus, and cellular response to organonitrogen compounds. The significantly enriched cellular components with high intensity include the plasma membrane region, the integral component of the plasma membrane, and the intrinsic components of the plasma membrane. The highly enriched molecular functions with high intensity include the catalytic activity acting on a protein, enzyme binding, and protein tyrosine kinase activity. The summary is represented in Supplementary Figure 3.

Among the top-enriched pathways associated with the cervical cancer gene targets are the pathways in cancer, cAMP signaling pathway, microRNAs, apoptosis, *HIF-1* signaling pathway, and *P13K-AKT* signaling pathway (Supplementary Table 3). Further pathway analysis depicts the role of the treatments in Environmental Information processing through the *HIF-1* signaling pathway, and the *P13K-AKT* signaling pathway, cellular processes in apoptosis, and in human diseases higher association in cancer, as shown in Supplementary Figure 4.

Molecular docking

Molecular docking was used to putatively validate the top 24 hub genes identified using four scoring parameters, as shown in Figure 7C, filtered from the protein network. The genes were docked against ivermectin, ascorbic acid, and the native ligand co-crystallized with the protein for docking validation (supplementary Table 4). Spontaneous binding between ligand and receptor is denoted by a binding energy of less than 0 kcal/mol. A binding energy of less than -5.0 kJ mol^{-1} denotes stronger binding between the protein and the ligand, while binding of less than -7.0 kJ mol^{-1} indicates a favourable and exceptionally strong binding of the ligand to the target protein [12]. Ivermectin was docked with the top 24 hub genes, and most of them had a favorable binding energy (-6.0 kJ mol^{-1} - -10.2 kJ mol^{-1}). These include *STAT3, TP53, BCL-2, MAPK1, PIK3CA, ILB1, BCL2L1, PTGS2, GSK3B, TLR4, HSPA8, MDM2, HIF1-α, MMP2, MMP9, PPARG, HSP90AA1, HSP90AB1,* and, *MCL1*. Interestingly, *TP53, IL1B, GSK3B*, and *TLR4* had lower binding scores than the co-crystalized ligand, suggesting stronger binding between ivermectin and the receptors. On the other hand, ascorbic acid had favourable binding energy -5.0 kJ mol^{-1} - -6.6 kJ mol^{-1} when docked with *STAT3, BCL2, MAPK1, PIK3CA, IL1B, BCL2L1, PTGS2, TLR4, HSPA8, JAK2, HIF1-α, MMP2, MMP9, PRKACA, PPARG, MCL1, HSP90AA1, and HSP90AB1*. Their interactions were mainly through hydrogen bonds and hydrophobic interactions. *TNF* was an interesting gene to explore its expression, given that it was the top hub gene across three scoring criteria (closeness, degree, and MNC) and that its binding energy with ivermectin was favorable (-6.9 kJ mol^{-1}). Representative 3D images of the docking are shown in Figure 7.

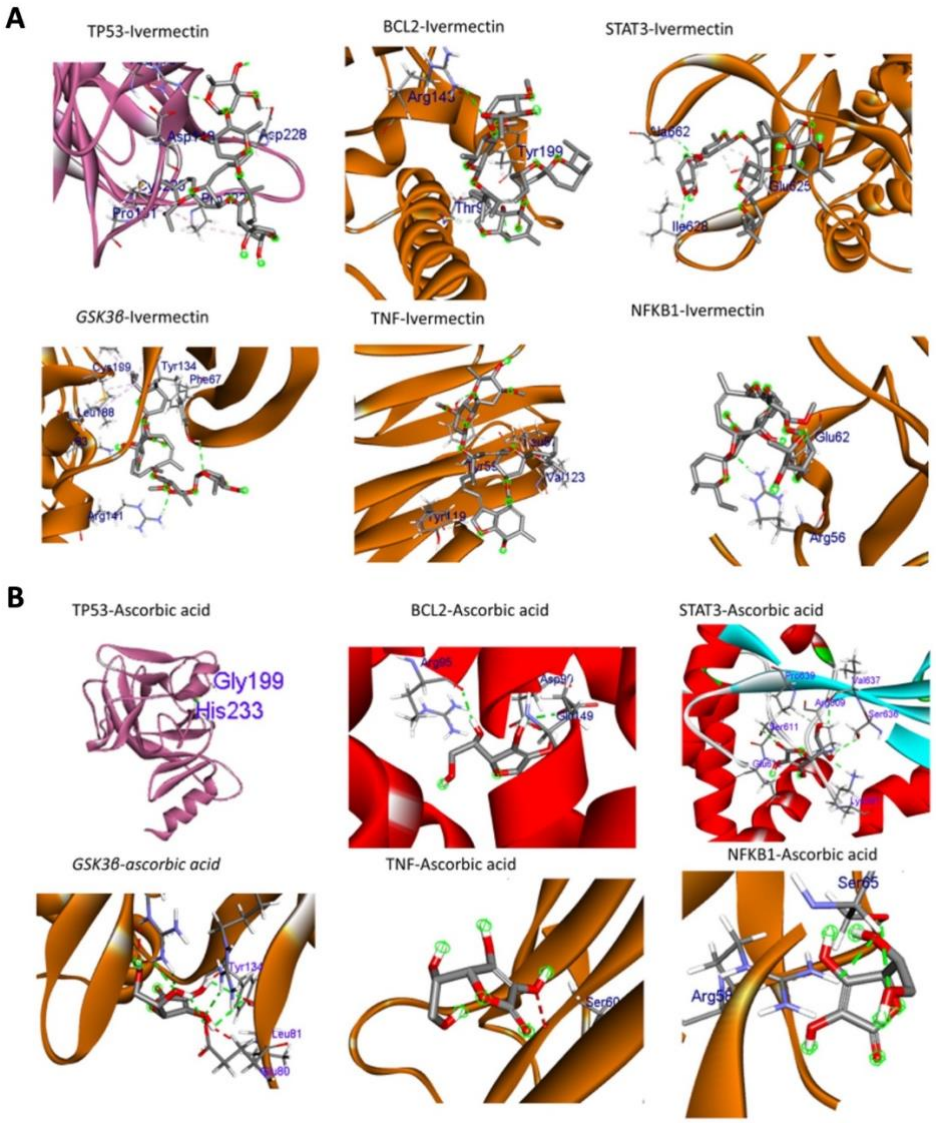

Figure 7. Molecular docking results. (A and B) show the 3D docking results of A) ivermectin and B) ascorbic acid, respectively, with TP53, BCL2, STAT3, GSK3β, TNF, and NFKB1. The 3D images were obtained after docking using PyRx software, followed by docking analysis using Discovery Studio Visualizer 2025.

Effect of ivermectin and ascorbic acid combination on mRNA expression of selected genes in HeLa cells

Gene expression analysis was performed to functionally validate the molecular docking results of the putative genes targeted by ascorbic acid and ivermectin in cervical cancer. We evaluated the expression of *TP53*, *BCL2*, *STAT3*, *TNF*, and *HIF1-α* in treatments containing ivermectin, ascorbic acid alone, or their combination, given the roles of these targets in apoptosis, hypoxia, and CSC-related pathways. We determined the cycle threshold (ct) and relative gene expression using *GAPDH* and β-actin as normalization genes. Our results show significant upregulation of *TP53* ($p<0.0286$) in HeLa cells treated with ivermectin. Notably, the expression of *TP53* in ascorbic acid was downregulated. There was a significant reduction in the expression of *STAT3* ($p<0.0001$), *BCL2* ($p<0.0109$), and *HIF1-α* ($p<0.0002$), as shown in Figure 8. *TNF* was significantly downregulated in all the treatments ($p<0.003$).

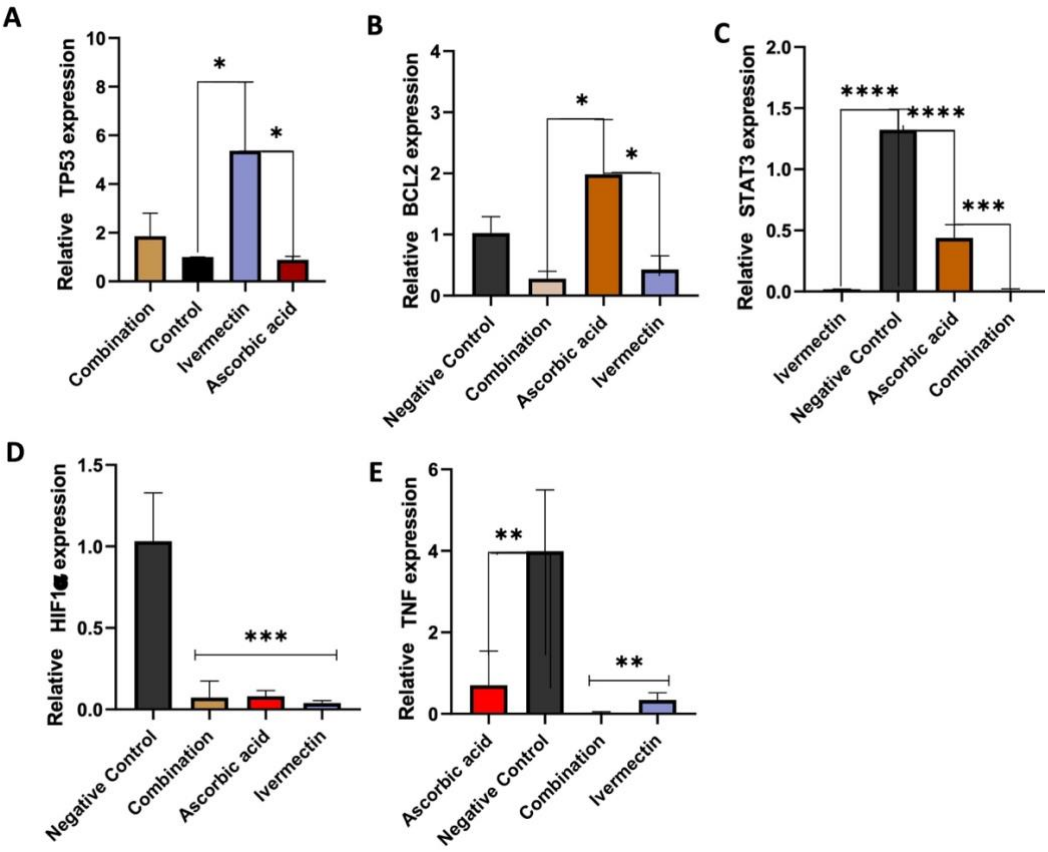

Figure 8. A-E) shows the relative gene expression of *TP53, BCL2, STAT3, HIF1-α,* and *TNF* genes, respectively, using *GAPDH* and *β-actin* as housekeeping genes when treated by a combination of ivermectin and ascorbic acid or individual drugs alone. The gene expression analysis was performed by comparing each treatment with the negative control using ANOVA, and the statistical significance is denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. A minimum of three independent experiments was performed for the mean and SD values.

DISCUSSION

Drug development for cervical cancer treatment is critical, especially for areas that are heavily burdened with the disease. In this exploratory proof-of-concept work, we adopt a drug repurposing approach and test for potential synergy between ivermectin and ascorbic acid. We therefore tested the antiproliferative activity of the individual drugs and in combination against cervical cancer HeLa cell lines using the MTT assay. We found the IC_{50} values for ivermectin and ascorbic acid after 48 hours of incubation to be ~9.64 μM and ~4.58 mM, respectively. These results are consistent with previous studies of anticancer effects of ivermectin and ascorbic acid [29,40]. The millimolar dosage of ascorbic acid is reported to only be achievable clinically through the intravenous route (IV) [26]. Thus, for this combination, further inquiry into an injectable formulation of the drug is warranted.

The combination showed additive effects at the IC_{50} and synergistic effects at higher doses. The observed synergy at higher doses is important with regard to cancer treatment [30]. Furthermore, analysis of the Dose Reduction Index (DRI) showed a favourable DRI at higher fa (fraction affected) values. This shows that the synergy observed at higher doses results in a significant reduction in the dose of both compounds. Notably, to our knowledge, this is the first experiment to report the interaction of ascorbic acid and ivermectin in HeLa cells. Ivermectin use in cervical cancer treatment has been debatable due to the attainment of *in vitro* concentration *in*

vivo [40]. Our findings on favourable dose reduction of individual compounds could address this gap through the synergy that provides efficacy at lower ivermectin concentrations. Human subjects subjected to an ivermectin dose of 2mg/kg were found to achieve a concentration of 5.2 μM [41]. We hypothesize that this combination treatment with ascorbic acid will reduce the required concentration to attain a clinically achievable ivermectin concentration, enabling its translatability to clinical practice.

The effects of the combination treatment and individual treatment on morphological changes in HeLa cells showed that cells exhibited dose-dependent reductions in viability and detachment, with irregular, rounded shapes, compared to the negative control (untreated cells), which maintained a consistent monolayer of cells with regular shapes. The effects of ivermectin on morphological changes in HeLa cells have been reported and are consistent with our results [42]. In ascorbic acid treatment, the cells had reduced proliferation and detachment, which is consistent with studies on the effect of ascorbic acid in inhibiting adhesion of cells [43].

Advanced cervical cancer stages are characterized by metastatic cells that migrate, colonize, and proliferate in distant organs in the body [12]. In this work, we evaluated the impact of the combination treatment on wound healing, a common approach to studying the effect of drugs in metastases [12]. As compared to the individual treatments, the combination had an antimetastatic effect similar to that of the individual compounds. These results are consistent with the results on the effect of high-dose ascorbic acid in inhibiting cell migration and ivermectin's effect on wound healing [43,44]. These results show that the combination treatment benefits from the individual drugs' abilities to reduce cell proliferation, induce detachment, and inhibit cell migration.

The potential of combination treatment was interrogated to reduce clonogenic potential using a colony formation assay, an in vitro technique used to evaluate the capacity of a single cell to self-renew [45]. Our study shows that the combination effectively eradicates single cells and prevents colony formation, compared to ivermectin, whose efficacy in eradicating all single cells was only at the IC_{50}. The combination behaved similarly to ascorbic acid in preventing colony formation, demonstrating its potency in effectively reducing colony formation. Preventing colony formation indicates the ability to target CSCs, though further tests using CSC markers and a sphere formation assay would provide more mechanistic insights [46]. Additionally, the ability of ascorbic acid to target CSCs has been previously reported in work on ascorbic acid and CSCs [15,20,21,43]. Thus, in the combination treatment, ascorbic acid may have contributed more to its ability to prevent single cells from proliferating into colonies.

To further interrogate the mechanism of antiproliferative activity of our treatment, we performed a DNA fragmentation assay as a functional surrogate assay to assess apoptosis. This method is easy and cost-effective for determining cell death and is suitable for detecting apoptosis in mammalian cells [47]. Our results show DNA fragmentation in the combination and individual treatments, with greater fragmentation in the combination, suggesting extensive DNA damage, which is characteristic of apoptosis [42]. This is concurrent with the results of apoptotic effects of ascorbic acid and ivermectin against cervical cancer HeLa cell lines [29,40]. The effects of ivermectin on DNA fragmentation reported in this work are consistent with other studies of ivermectin-induced DNA damage in HeLa cells [42]. However, further confirmatory mechanistic assays using Annexin V/PI staining or caspase activation are required to additionally support this finding.

Further analysis using computational approaches, network pharmacology showed that ivermectin and ascorbic acid have 84 shared targets, 92 unique target genes for ascorbic acid, and 133 unique target genes for ivermectin in cervical cancer. Shared targets in a gene network would be one of the possible reasons for synergy between two drugs [27]. Pathway analysis using the KEGG platform found that the compounds influenced key pathways in cervical cancer, including pathways in cancer (hsa05200), MicroRNAs in cancer (hsa05206), Chemical carcinogenesis-receptor activation (hsa05207), apoptosis (hsa042100), *HIF-1* signaling pathway (hsa04066), and *P13K-AKT* signaling pathway (hsa04151). The *P13K-AKT* signaling pathway is associated with metastasis, cancer cell proliferation, drug resistance, and differentiation [28]. The *HIF-1* signaling pathway is associated with the onset of tumorigenicity, drug resistance, metabolic reprogramming, angiogenesis, cell invasion and migration, and cancer cell survival [48]. These findings provide preliminary insights into the mechanism of action of the combined treatment in targeting various pathways necessary for the progression of cervical cancer.

Further protein network analysis of the common targets between ivermectin, ascorbic acid, and cervical cancer identified 24 hub target genes. Among the 24 top hub genes, *TNF, STAT3,* and nuclear factor kappa B subunit 1 (*NFKB1)* have been implicated in establishing resistance in cancer stem cells [49,50]. *TNF* has also been implicated in mediating stemness in cancer cells through the Notch-Hes1 pathway [51,52]. *TP53* and *BCL2* are well-established genes that control apoptosis in cells [53,54]. Influencing the regulation of these two genes would lead to cell death in cervical cancer cells, predicting the potential of our treatment to function mechanistically by targeting them. Network pharmacology also pointed to the potential of targeting *HIF1α*, a known driver of carcinogenesis in hypoxic environments [55]. Peroxisome proliferator-activated receptor gamma (*PPARG*) plays a role in modulating metabolic reprogramming, mitochondrial biogenesis, metastases, growth, and survival in cancer cells. Though its role in cancer is context-based, its overexpression in cervical cancer is associated with cell growth, tumorigenesis, and drug resistance [37]. Glycogen synthase kinase 3β (*GSK3β*) is involved in energy metabolism, mitochondrial dysfunction, inflammation, and apoptosis, where in cervical cancer, it was found to activate the *P13K-AKT* signaling, reducing cell viability [56]. Therefore, these results from network pharmacology predict the potential of our combined treatment in targeting apoptosis, cancer stem cells, metabolism, and hypoxic environments.

The top hub genes were then subjected to molecular docking with ivermectin and ascorbic acid. The results show favourable binding energy of ivermectin ranging between -6.0 kJ mol^{-1} and -10.2 kJ mol^{-1} and -5.0 kJ mol^{-1} - -6.6 kJ mol^{-1} for ascorbic acid for the top hub genes. These results show that the compounds effectively target most of the hub genes since a docking score less than -5.0 kJ mol^{-1} shows a desirable binding affinity [12,28]. The bonds established between the compounds were mainly hydrogen bonds and hydrophobic interactions.

Experimental validation of the molecular docking results was performed using qPCR gene expression analysis, in which we analysed the expression of *TP53, STAT3, BCL2, TNF*, and *HIF1α* due to their identified roles in apoptosis, CSC-mediated tumour growth, and hypoxia, as well as their favourable binding energies following molecular docking. *TP53* was found to be upregulated following gene expression analysis in ivermectin compared to the negative control ($p<0.0286$). Its expression in the combination was upregulated, without a significant difference from the negative control, which may be due to the low ivermectin concentration. There was no significant difference in *TP53* expression between ascorbic acid and the negative control.

This suggests that the cell death mechanism in ascorbic acid may differ, potentially contributing to reduced antiproliferative activity in the combination [29].

The current study shows that *BCL2* was significantly down-regulated in the combination treatment and in ivermectin ($p<0.0109$), but not in ascorbic acid treatment. Studies have demonstrated ivermectin's role in targeting *BCL2* and overcoming *BCL2*-mediated apoptotic resistance in lung cancer cells [57]. Thus, these results suggest the potential of targeting mitochondrial metabolism and apoptotic pathways with this combination treatment. *STAT3* expression was downregulated in both treatments as compared with the negative control ($P<0.0001$).

In this study, we found *HIF1α* to be downregulated in all treatments when compared to the negative control ($p<0.0002$). There were no differences among the treatments ($p>0.999$). *TNF* was downregulated in all treatments with no significant difference between treatments ($p>0.9547$) but with a significant difference with the negative control ($p<0.003$). These findings suggest a role for the combination treatment in modulating apoptosis, CSC-related pathways, and hypoxia; however, protein-level validation is necessary to support these findings. Taken together, our present combination treatment shows promising results of using ivermectin and ascorbic acid in cervical cancer treatment. Such findings are relevant in the context of LMICs, where accessibility and affordability of advanced anticancer drugs are still a major health issue.

The limitation of the current study is the use of predictive computational approaches and the lack of additional functional assays to demonstrate the cell death mechanism and the CSC-targeting. This can be achieved using advanced methods that integrate multiomics and patient-derived 3D models, which can provide the ideal framework for CSC heterogeneity [58]. Future experiments to advance this work should consider testing for apoptosis using Annexin V staining, testing for cell cycle arrest using flow cytometry, and western blotting for protein level validation. We also recommend future testing using additional cervical cancer-associated cell lines, such as SiHa, CaSki, and C33A cell lines, among others, to cover various subtypes of cervical cancer. Further studies should also explore the *in vivo* bioactivity of the combination and possible formulations.

CONCLUSIONS

In this proof-of-concept exploratory study to determine the potential use of ivermectin and ascorbic acid for cervical cancer treatment, the study shows that there is a dose-dependent antiproliferative activity of a combination of ivermectin and ascorbic acid against cervical cancer HeLa cells. Moreover, there was an additive effect at the IC_{50} concentration of the combination of the two drugs. This data provides preliminary insights into the interactions between ascorbic acid and ivermectin in the treatment of cervical cancer (Figure 9). This combination treatment approach has reduced the individual dosages of the drugs, and it was predicted to target drivers of cervical cancer and the stem cells through critical pathways like the *P13K-AKT* signaling pathway. In conclusion, this exploratory study shows dose-dependent antiproliferative activity of the combination treatment and suggests weak synergism between ivermectin and ascorbic acid in cervical cancer treatment.

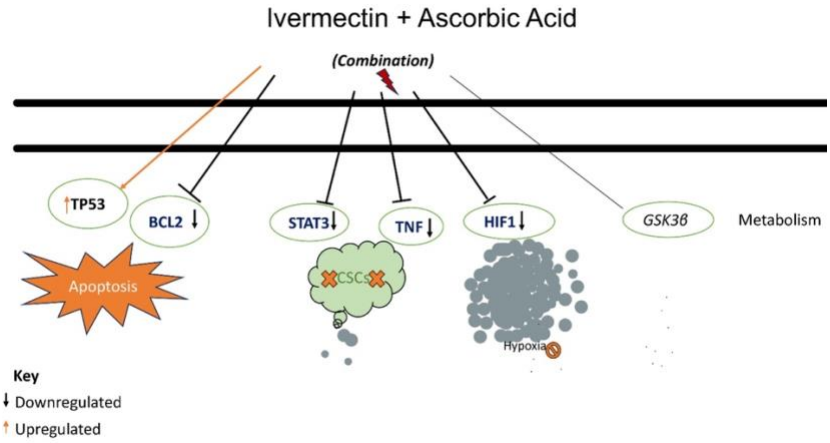

Figure 9. Proposed mechanism of action of the combination of ivermectin and ascorbic acid against cervical cancer based on gene expression of TP53, STAT3, BCL2, TNF, and HIF1α. The potential role of GSK3β in controlling cervical cancer metabolism is predicted using network pharmacology and KEGG pathway analysis.

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AUTHOR CONTRIBUTIONS

Conceptualization, SOO, MCK, JK, ENM, and SNN; Methodology, SOO, MCK, JK, ENM, and SNN; Software, SOO and SNN; Validation, SOO and SNN Formal Analysis, SOO and SNN; Investigation, SOO, MCK, JK, ENM and SNN; Resources, SOO; Data Curation, SOO and SNN; Writing – Original Draft Preparation, SOO; Writing – Review & Editing, SOO, MCK, JK, ENM and SNN; Visualization, SOO, MCK, JK, ENM and SNN; Supervision, MCK, JK, ENM and SNN; Project Administration, MCK; Funding Acquisition; N/A. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

SUPPLEMENTARY MATERIALS

Supplementary Figure 1. Effect of ivermectin, ascorbic acid, and their combination on the curve, plot of dose reduction index, and logarithmic plot of the combination Index, Supplementary Figure 2. A Venn diagram showing common gene targets for cervical cancer genes and predicted targets for ivermectin and Ascorbic acid, Supplementary Figure 3. GO and KEGG enrichment analysis, and Supplementary Figure 4. Pathway analysis is associated with ivermectin and ascorbic acid targets in cervical cancer. Supplementary Table 1. Diagonal combination method as described by Chou and Talalay, Supplementary Table 2. Top 30 hub genes targeted by ascorbic acid and ivermectin in cervical cancer, Supplementary Table 3. Top 20 enriched pathways from

KEGG analysis of pathways associated with ivermectin and ascorbic acid in cervical cancer, and Supplementary Table 4. Molecular docking data. (Supplementary materials).

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