RESEARCH ARTICLE

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Anti-viral and Apoptotic Induction of m-TOR Inhibitor Drugs against Hepatitis C Virus Activity and Hepatocellular Carcinoma Cell Line: *In vitro* and *in silico*

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Abstract

Objective: This study investigated the potential of m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) in combating both hepatocellular carcinoma (HCC) and hepatitis C virus (HCV) replication. **Methods:** After treating HepG2 and PBMCs with the mammalian target of Rapamycin (m-TOR) inhibitors drugs; sirolimus, everolimus, and tacrolimus at different concentrations (1, 5, and 10 µM/µl), cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Antioxidant activities (total antioxidant, glutathione S-transferase, and glutathione reductase), Fas-ligand level, tumor necrosis factor-α (TNF-α) level, caspase-3, -8, and -9 activities, and cell cycle analysis were measured. quantitative Real-time PCR, colony forming assay, molecular docking studies after infection of PBMCs with 1 ml (1.5×10^6 HCV) serum then incubated with m-TOR inhibitor drugs at their respective IC50 concentrations. **Results:** In HepG2 cells, treatment with these inhibitors resulted in suppressed cell viability, increased dead cell accumulation, and enhanced apoptotic signaling through elevated Fas-ligand and caspase activities. Additionally, cell cycle analysis revealed arrest in G0/G1 and G2/M phases, further hindering HCC progression. Furthermore, m-TOR inhibitor drugs significantly reduced HCV viral load and colony formation in infected PBMCs. This antiviral effect was accompanied by decreased TNF-α activity, suggesting potential modulation of the inflammatory response associated with HCV infection. Molecular docking studies provided theoretical support for these findings, with Sovaldi demonstrating the highest binding affinity towards key HCV targets compared to other m-TOR inhibitors. This suggests its potential as a potent HCV inhibitor, while also highlighting the potential of exploring m-TOR inhibitors for future HCV treatment development. **Conclusion:** Overall, this study provides encouraging evidence for the potential of m-TOR inhibitor drugs as promising therapeutic agents for both HCC and HCV, warranting further investigation and optimization for clinical applications.

Keywords: m-TOR inhibitor drugs- HCV- HCC- Docking

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Introduction

Hepatocellular carcinoma (HCC), a type of liver cancer, looms large as a global health concern. According to the latest data from the International Agency for Research on Cancer, 2020 saw an estimated 906,000 cases and 830,000 deaths worldwide, making it a leading cause of mortality [1]. The shadows of this disease fall on many, highlighting the urgent need for effective prevention and treatment strategies. While the causes of HCC vary, some common culprits stand out. Chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) can silently chip away at liver health, eventually leading to cancer [2]. Contaminated food harboring aflatoxin, a potent carcinogen, also poses a significant threat. Additionally, lifestyle factors like heavy alcohol consumption, non-alcoholic fatty liver disease (NAFLD), excess weight, and smoking can all contribute to the development of HCC [2]. Understanding these triggers and exploring new treatment options are crucial steps in the fight against HCC. This study delves into the potential of mTOR inhibitor drugs – sirolimus, everolimus, and tacrolimus – as weapons against this formidable foe. We investigate their effects on both HCC

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and HCV, aiming to shed light on their potential role in combating these interconnected adversaries. Hepatitis C virus (HCV) infection is a global health concern, affecting 71.1 million people globally, or 1% of the total population, with chronic infection [3, 4]. Hepatocyte transplantation is the final stage of treatment for people with hepatitis C virus-associated liver cancer (HCV-HCC), which may contribute to the initial treatment of early cases of liver cancer, but recurrence associated with liver transplantation will become a major problem in the coming years [5, 6]. There are many pathways inside the cells that control the incidents inside the cells and deregulation of these pathways leads to increase proliferation of the cells and transform it to cancer cells. One of the most vital of these pathways is the m-TOR pathway. M-TOR signaling pathway incorporates both intracellular and extracellular signals where, it regulates cellular metabolism, growth, and proliferation and several downstream pathways that regulate cell cycle progression [7]. At the molecular level, HCC development including chronic HCV showed that associated with m-TOR pathway [8]. HCV nonstructural proteins 5A (NS5A) and 5B (NS5B) are multifunctional proteins that play crucial roles in the HCV replication cycle and modulation of host cell responses [9]. NS5A activates m-TOR pathway by triggering the phosphorylation of two m-TOR targeted proteins, S6K1 and 4EBP1 which results in increasing the replication and pathogenesis of HCV and also leads to suppression of apoptosis in Huh7 cells [10]. The regulation of NS5B phosphorylation can impact its activity and function in the viral replication process. According to *in vitro* kinase experiments, PKN2 phosphorylates the HCV NS5B protein alone. When endogenous PKN2 is knocked down, HCV RNA replication is inhibited. The phosphorylation happens through contact with the N-terminal finger domain of NS5B, which is composed of amino acids 1-187 of the protein [11]. In another study, it was shown that knocking down PKN2 decreased both basal and insulin-stimulated phosphorylation of m-TOR, resulting in a decrease in the signaling of m-TOR and its downstream protein synthesis [12]. While liver transplantation offers hope for many with end-stage liver disease, potent immunosuppressive drugs are crucial to prevent organ rejection. M-TOR inhibitors like sirolimus, everolimus, and tacrolimus have established roles in this context, suppressing T-cell activity and cytokine production [13]. However, their potential extends beyond immunosuppression, with growing evidence suggesting anti-cancer properties against various tumors, including hepatocellular carcinoma (HCC) [14]. Several studies have delved into the mechanisms behind these anti-cancer effects. Everolimus, for instance, binds to FKBP-12, inhibiting m-TOR signaling and subsequently T cell proliferation and antibody production [15]. Sirolimus, on the other hand, impedes T-cell progression through various pathways, even suppressing the growth factors IL-12, IL-2, and IL-4 [14, 16,17]. In contrast, tacrolimus, a macrolide calcineurin inhibitor, blocks the activation of NF-AT, a key transcription factor for cytokine genes like IL-2 [18, 19]. Despite their immunosuppressive nature, numerous studies highlight the anti-cancer potential of these mTOR inhibitors across various cancer cell lines, including HCC [20-22]. This has sparked our interest in exploring their dual role in the context of HCC and hepatitis C virus (HCV) infection.

Materials and Methods

M-TOR inhibitor drugs

Mammalian target of rapamycin (m-TOR) inhibitor drugs are prepared from, these are three types of drugs with the appearance as white to off-white crystalline powder, where they are soluble in DMSO (1 %). Rapamune (Sirolimus, rapamycin) 1 mg (LKT-R0161-M001), everolimus 1 mg (LKT-E8419-M001), tacrolimus 1 mg (LKT-T0008-M001).

Hepatocellular carcinoma (HepG2) and isolation of peripheral blood mononuclear cells (PBMCs) by Ficol Separation Media

This study utilized two cell types crucial for investigating hepatitis C (HCV) infection: hepatocellular carcinoma cells (HepG2) and peripheral blood mononuclear cells (PBMCs). HepG2 cells: The HepG2 cell line, obtained from the National Cancer Institute in Cairo, Egypt, serves as a well-established model for studying liver cancer. HepG2 cells were maintained as previously mentioned [23], the cells were cultured in RPMI 1640 media (Sigma-Aldrich in St. Louis, Missouri, USA), and incubated at 37°C in a 5% CO_2 incubator. Some studies have suggested that HCV replication in PBMCs might contribute to the persistence of the virus in the body and the ability to evade the immune response [24, 25]. Therefore, PBMCs, isolated from healthy donors using standard Ficoll separation techniques (BiochromAG, Berlin, Germany) [26], represent the immune system's frontline defense against infections. Rigorous quantitative real-time RT-PCR testing confirmed the absence of any prior HCV infection in these donated PBMCs, ensuring a clean starting point for further experiments [26, 27]. Fetal bovine serum (FBS) was added to the PBMC culture medium as a source of nutrients, supporting their growth and function.

Assessing Cell Viability and Response to m-TOR Inhibitors: The Power of the MTT Assay

This study delved into the effects of m-TOR inhibitor drugs on cell viability and response, utilizing the versatile MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as our investigative tool [23, 28]. Experimental procedures: Seeding the Stage: Both HepG2 and PBMC cell lines were meticulously placed in 96-well plates, with each well harboring 7,000 to 10,000 cellular residents. These miniature communities were then nurtured in RPMI 1640 medium enriched with 10% FBS for 48 hours, allowing them to adapt and thrive [28]. Treatment Time: After this initial growth period, the old medium was gently removed, and the cells were refreshed with PBS. The real test began as designated wells received varying concentrations (1, 5, and 10 µM/µl) of each m-TOR inhibitor drug, exposing the cells to their potential influence for 48 hours in the case of HepG2 cells [23]. MTT Unveils the Story: To assess how the cells

fared under these treatments, we employed the MTT assay. This involved adding a small amount of MTT solution to each well, followed by a 4-hour incubation at 37°C. During this time, metabolically active cells within the well converted the MTT into a colored formazan product [23]. Quantifying the Impact: Finally, the formazan product was dissolved, and its absorbance at 570 nm was measured using a specialized plate reader. This numerical value served as a proxy for cell viability, allowing us to calculate the half-maximal inhibitory concentration (IC_{50}) and overall percentage of viable cells using GraphPad Prism 7 software [23].

Unveiling the Antioxidant Potential of m-TOR Inhibitors in HepG2 Cells

This study explored the intriguing possibility that m-TOR inhibitor drugs – sirolimus, everolimus, and tacrolimus – might also possess antioxidant properties within HepG2 cells. To test this hypothesis, cells were treated with each drug at their IC_{50} concentrations for 48 hours. We then meticulously measured the activity of three critical antioxidant players: total antioxidant capacity (TAC), glutathione S-transferase (GST), and glutathione reductase (GR). To ensure accuracy and consistency, we employed colorimetric assay kits from Biodiagnostic (Giza, Egypt) and meticulously followed the manufacturer's instructions.

Apoptotic Potential of m-TOR Inhibitors in HepG2 Cells: A Key Protein Investigation

This study investigated the potential of three m-TOR inhibitor drugs - sirolimus, everolimus, and tacrolimus - to trigger programmed cell death (apoptosis) in HepG2 liver cancer cells. The researchers focused on changes in key proteins involved in the apoptotic pathway, specifically Fas-Ligand, Caspase-3, -8, and -9. These proteins play crucial roles in initiating and executing the cell death cascade. HepG2 cells were treated with each drug at its half-maximal inhibitory concentration (IC_{50}) for 48 hours. Activity levels of Fas-Ligand were measured using an enzyme-linked immunosorbent assay (ELISA) kit from Wkea Med Supplies (Changchun, China), while caspase activities were assessed using specific colorimetric assay kits from commercial suppliers. By analyzing these protein activities, the research aimed to determine whether m-TOR inhibitor drugs could induce apoptosis in HepG2 cells. This finding could open up new avenues for cancer therapy targeting liver cancer.

Examination of HepG2 cell morphology after treatment with m-TOR inhibitor drugs

Researchers investigated the physical effects of m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) on HepG2 cells, examining changes in cell appearance. Cells were treated with individually determined half-maximal inhibitory concentrations (IC_{50}s) of each drug for 48 hours and incubated at 37°C with 5% CO2. Following incubation, researchers used a Leica DMi8 inverted microscope (Leica Microsystems) with 20x magnification and Leica Application Suite X (LAS X) software to analyze cell morphology. This analysis aimed to identify any visible changes in cell shape, size, or other characteristics caused by exposure to the m-TOR inhibitors.

Cell cycle analysis

To understand how m-TOR inhibitor drugs affect cell division in HepG2 cells, researchers utilized the Cytell™ Cell Cycle Kit from GE Healthcare Japan (Tokyo, Japan). Cells were treated with individually determined half-maximal inhibitory concentrations (IC_{50}s) of sirolimus, everolimus, and tacrolimus for 48 hours followed by incubation at 37°C and 5% CO2. Subsequently, the Cytell™ cell imaging system from GE Healthcare Japan (Tokyo, Japan) was used to analyze stained cells, revealing the distribution of cells across different phases of the cell cycle (G1, S, G2/M). This analysis aimed to determine if these drugs impact cell proliferation by altering how cells progress through the cell cycle.

PBMC infection with HCV serum and treatment with m-TOR inhibitor drugs

Millions of immune cells, or PBMCs, were cultured in a sterile dish at a density of one million per milliliter. To simulate a hepatitis C virus (HCV) infection, researchers exposed these cells to a specific dose of the virus (1.5 million copies) for 3 hours. Following this initial exposure, the PBMCs were allowed to recover for 24 hours in a special nutrient medium resembling human body (RPMI 1640 medium from Sigma-Aldrich, St Louis, MO, USA), the condition (37°C with 5% CO_2). Next, to explore potential treatment options, researchers meticulously washed and divided the PBMCs into groups. Each group received one of three m-TOR inhibitor drugs: sirolimus, everolimus, or tacrolimus, administered at their most effective concentrations for inhibiting cell growth. For 48 hours, the treated PBMCs continued to incubate under controlled conditions, mimicking the human body's environment.

Finally, to assess the effectiveness of the drugs, researchers carefully extracted genetic material from the PBMCs. Using a highly sensitive technique called quantitative real-time PCR, they precisely measured the number of remaining virus copies within each cell. This analysis aimed to determine whether the m-TOR inhibitor drugs helped the PBMCs combat the HCV infection by reducing the viral load.

Total RNA Extraction and Quantitative Real Time RT-PCR (qRT-PCR)

To measure the impact of m-TOR inhibitor drugs on hepatitis C virus (HCV) infection, researchers carefully extracted RNA from PBMCs using a specialized QIAamp® RNA Blood Mini Kit (QIAGEN). This was performed both before and after treating the cells with sirolimus, everolimus, and tacrolimus, adhering strictly to the kit's instructions. The quality and quantity of extracted RNA were then evaluated using a NanoDrop™ instrument, confirming its suitability for further analysis. Next, the HCV quantitative Real Time PCR Kit was employed to determine the precise number of HCV RNA copies present in the supernatant of infected and treated PBMCs.

As with the RNA extraction, meticulous adherence to the manufacturer's instructions was ensured. Finally, samples were analyzed on a sophisticated 7500 fast Real-Time PCR System (Applied Biosystems) running a 40-cycle test. By comparing the results to a pre-established standard, researchers could estimate the amount of HCV virus present, akin to how a doctor might use the CobasAmpliPrep/CobasTaqMan HCV test to gauge viral levels in patients.

Colony Forming Assay for estimation of HCV replication in infected untreated and treated PBMC with m-TOR inhibitor drugs

Pre-clinical assessment of antiviral drugs often utilizes colony-forming assays to estimate their efficacy by measuring colony formation. Briefly, PBMCs were isolated using cell separation media and cultured at a density of 1 x 10^6 cells/ml in sterile 15ml Falcon tubes. They were then infected with 1 ml (1.5 x 10^6 HCV) serum as previously described. Following infection, the cell density was adjusted to 1-2 x 10^5 cells per 24-well plate and treated with m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) at their respective IC_{50} concentrations. Subsequently, cells were washed with 1.0 M PBS (pH 7.4). Both untreated and treated infected PBMCs were stained with Coomassie blue (Coomassie® Brillia). Finally, morphological changes in each well were evaluated using an inverted microscope (Zeiss Axio Vert. A1, Zeiss, Göttingen, Germany) at 40x magnification and documented with the microscope's digital camera (Color Digital Imaging-SPOT Idea 3MP).

Assessment of TNF-α concentration in infected untreated and treated PBMC with m-TOR inhibitor drugs

Studies have shown that a molecule called tumor necrosis factor-alpha (TNF-α), also known as cachectin, is significantly elevated in people with chronic hepatitis C infection [27, 28]. In this experiment, we aimed to explore how m-TOR inhibitor drugs might influence the levels of this specific cytokine within infected immune cells.

Firstly, we isolated PBMCs and exposed them to a simulated HCV infection using 1 ml of HCV serum containing 1.5 million infectious units (UI), following the previously described protocol. Next, we divided the infected cells into groups and treated them with different m-TOR inhibitors (sirolimus, everolimus, and tacrolimus) at their most effective concentrations (IC_{50}s). To measure the resulting changes in TNF-α levels, we employed a specialized kit from KomaBiotech (Seoul, South Korea) known as ELISA Kit (K0331131P). This kit allowed us to precisely quantify the amount of TNF-α present within each cell sample, following the manufacturer's instructions to ensure accuracy. By analyzing these measurements, we hoped to gain insights into whether the m-TOR inhibitors could potentially modulate the immune response to HCV infection by regulating TNF-α production within infected PBMCs.

In silico study Docking Study of m-TOR inhibitor drugs

This investigation employed an *in silico* docking approach to assess the potential inhibitory effects of m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) and the established HCV treatment, Sovaldi, against key proteins of the hepatitis C virus (HCV). Specifically, the study targeted Nonstructural proteins 5A (NS5A) and 5B (NS5B), crucial for the viral life cycle, as well as Human protein kinase N2 (PKN2), which the virus exploits for replication.

The docking simulations were conducted using the Glide's Standard Precision (SP) module within Schrodinger 16.4 software. Ligand preparation involved Maestro 13.5 and LigPrep 2.4 software, with ligands obtained from the PubChem Bioassay database. Crystallographic structures of the target proteins were retrieved from the Protein Data Bank (PDB codes: PKN2-4CRS.pdb, NS5A-6P6Z.pdb, and NS5B-4KHM.pdb). Initially, a standard grid box size of 20 Angstroms was defined for each protein to encompass the search area for optimal drug-protein interactions.

This initial docking analysis constitutes the first step in this investigation. Subsequent evaluations will delve deeper into the predicted binding strength and specific details of these interactions, aiming to garner valuable insights into the potential of these drugs to disrupt critical HCV protein functions and contribute to developing effective antiviral strategies.

Statistical Analysis

To evaluate the statistical significance of observed differences, a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test was employed. This analysis, performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA), identified groups with statistically significant differences at a significance level of $p < 0.01$ and $p < 0.05$.

Results

Cytotoxic Effects of m-TOR Inhibitors on HepG2 Cells and PBMCs

This study assessed the cytotoxic effects of three m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) on both HepG2 cells and isolated PBMCs using the MTT assay at 48 hours post-treatment.

HepG2 Cells

All three m-TOR inhibitors significantly reduced HepG2 cell viability at both 10 μM/μl and 5 μM/μl concentrations ($p < 0.01$ and $p < 0.05$, respectively). Sirolimus exhibited the least impact, reducing viability by 27.7% at 10 μM/μl and 19.7% at 5 μM/μl. Conversely, everolimus demonstrated the strongest effect, decreasing viability by 37.7% at 10 μM/μl and 21.3% at 5 μM/μl. Tacrolimus showed intermediate potency, with reductions of 31.3% at 10 μM/μl and 17.7% at 5 μM/μl. Notably, no significant cytotoxicity was observed at the 1 μM/μl concentration for any drug. The determined IC_{50} values confirmed these observations, with values of 3 μM/μl, 1.3 μM/μl, and 2 μM/μl for sirolimus, everolimus, and tacrolimus, respectively (Figure 1A).

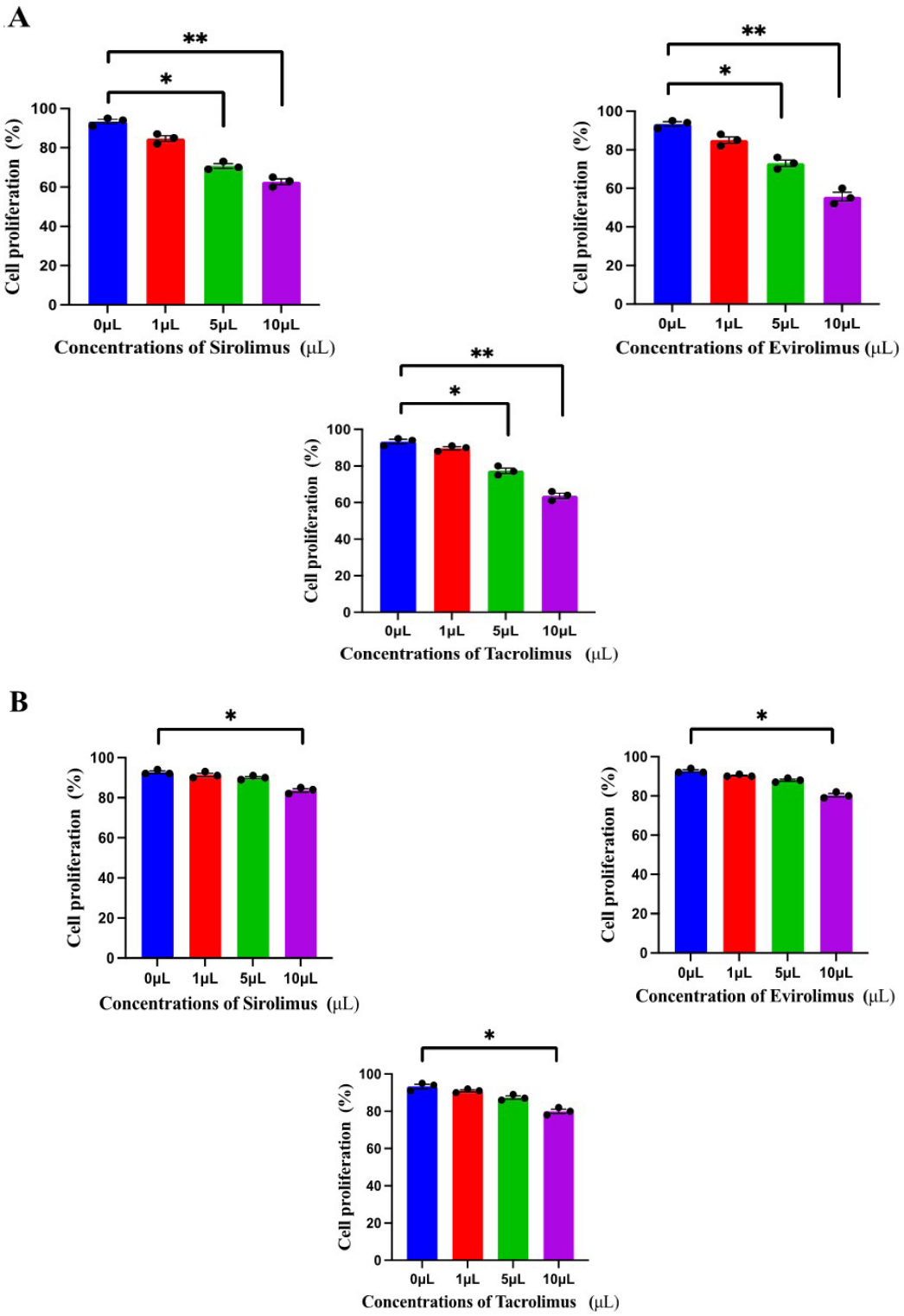

Figure 1. A) Cell viability of HepG2 cells treated with m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) at different concentrations (1, 5, and 10µl) compared to control untreated HepG2 cells. B) Cell viability of PBMC treated with m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) at different concentration (1, 5, and 10µl) compared to untreated PBMC. The mean of three independent replicates was used to calculate these data values. Data presented as mean±SD. (*) represents p-value < 0.05 and (**) represents p-value < 0.01 compared to control untreated HepG2 and PBMC cells.

PBMCs

Compared to HepG2 cells, all three m-TOR inhibitors displayed a weaker cytotoxic effect on PBMCs. Only the 10 µM/µl concentration caused a significant decrease in PBMC viability ($p < 0.05$), with reductions of 16%, 19%, and 20% for sirolimus, everolimus, and tacrolimus, respectively. Lower drug concentrations (5 µM/µl and 1 µM/µl) had no statistically significant impact on PBMC viability compared to untreated controls. Furthermore, the IC_{50} values for PBMCs were significantly higher than those for HepG2 cells, at 7.5 µM/µl, 7 µM/µl, and 6.8 µM/µl for sirolimus, everolimus, and tacrolimus, respectively

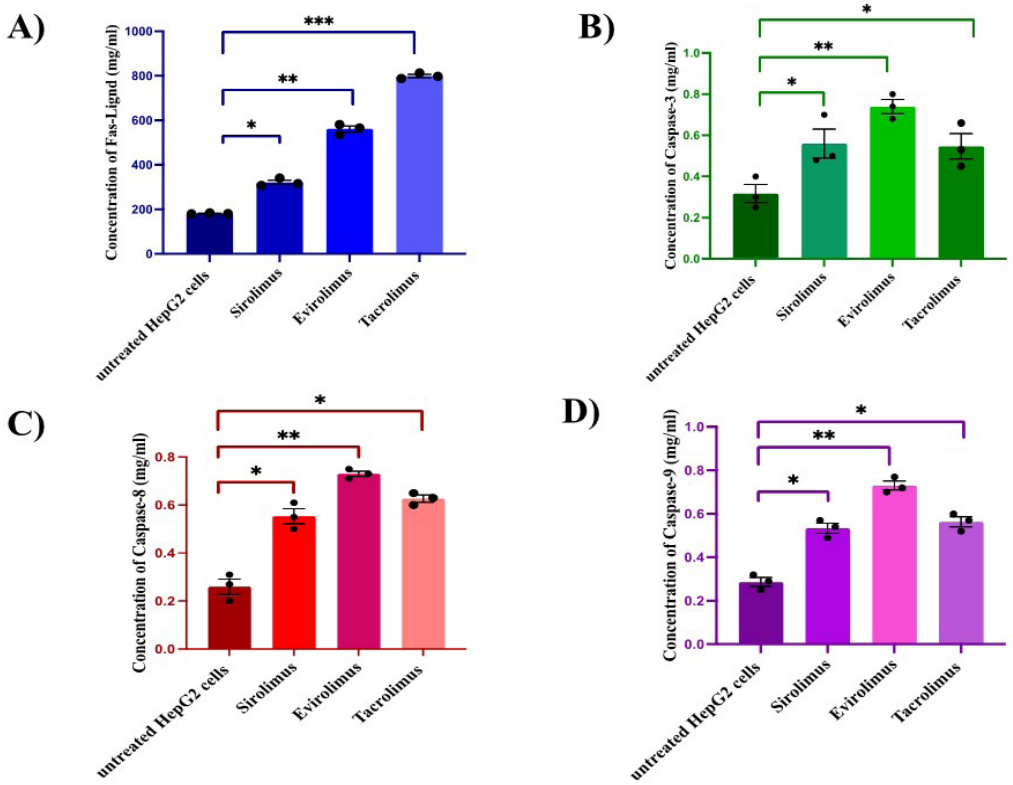

Figure 2. Quantification of the Changes in the Activity of A) Fas-ligand, B) Caspase-3, C) Caspase-8, and D) Caspase-9 in HepG2 cells following treating with sirolimus, everolimus and tacrolimus at IC_{50} concentration for 48 hrs. The mean of three independent replicates was used to calculate these data values. Data presented as mean ± SD. (*) represents p-value < 0.05 and (**) represents p-value < 0.01 compared to control untreated HepG2.

(Figure 1B). These findings suggest a stronger resistance of PBMCs to the cytotoxic effects of m-TOR inhibitors compared to HepG2 cells.

m-TOR Inhibitors enhance Antioxidant Capacity in HepG2 Cells

Following 48-hour treatment of HepG2 cells with m-TOR inhibitors at their respective IC_{50} concentrations, all three examined antioxidants – total antioxidant capacity, glutathione S-transferase (GST), and glutathione reductase – displayed significant increases ($p < 0.05$) compared to untreated cells. Notably, everolimus treatment resulted in the highest activity levels for all three antioxidants, surpassing both sirolimus and tacrolimus, as detailed in Table 1.

m-TOR Inhibitors induce apoptotic signaling in HepG2 Cells

Following 48-hour treatment of HepG2 cells with m-TOR inhibitors at their respective IC_{50} concentrations, the study investigated changes in apoptotic signaling pathways.

Fas-Ligand

Tacrolimus treatment resulted in the most dramatic upregulation of Fas-ligand activity, displaying a staggering 612.5-fold increase compared to untreated control cells ($p < 0.001$). This marked increase is clearly evident in Figure 2A. Everolimus also significantly elevated Fas-ligand activity, albeit to a lesser extent, with a 373.8-fold increase ($p < 0.01$) as shown in Figure 2A]. Sirolimus, compared to the other two inhibitors, induced a more modest but still significant 126.3-fold increase ($p < 0.05$). Among the analyzed caspases, everolimus treatment triggered the strongest activation. It induced increases of 0.4, 0.43, and 0.44-fold in caspases 3, 8, and 9, respectively, compared to controls ($p < 0.01$). These caspase activation levels are visually represented in Figures 2B, 2C, and 2D.

Both sirolimus and tacrolimus also demonstrably

Table 1. Total Antioxidant, Glutathione S-transferase and Glutathione Reductase Activities in HepG2 Cells Treated m-TOR Inhibitor Drugs at 37°C in a 5% CO_2 Incubator for 48 h.

Antioxidant	Control untreated HepG2 cells	Sirolimus treated HepG2 cells (3μM/μl)	Everolimus treated HepG2 cells (1.3μM/μl)	Tacrolimus treated HepG2 cells (2μM/μl)	Statistical significance
Total antioxidant, (μM/ml)	0.6±0.0	0.9 ±0.3	1.2±0.5	0.78±0.8	P<0.05
Glutathione S-transferase, (μM/ml)	382.1±61.0	485.7±22.0	517±21.0	470±41.0	P<0.05
Glutathione reductase, (mg/dl)	3.9±0.5	12.5±0.7	15.7±0.5	10.8±0.6	P<0.05

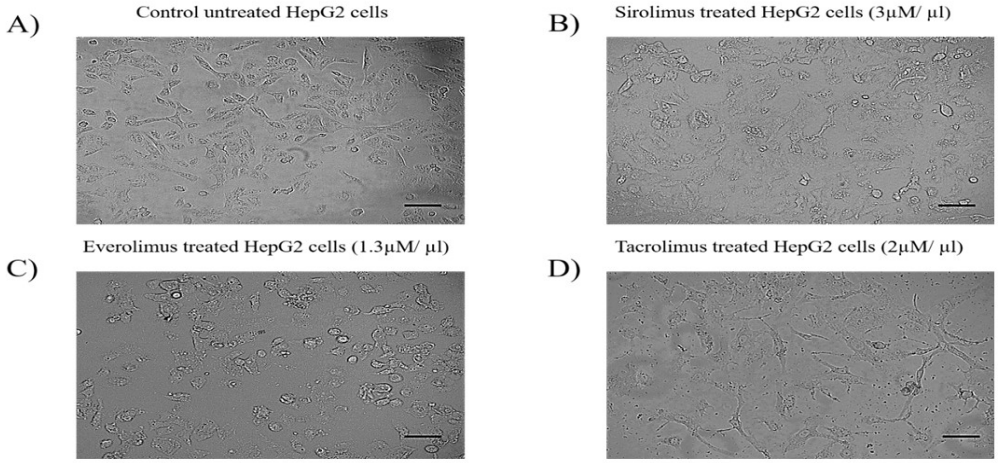

Figure 3. Representative Photomicrographs Showing the Viability of HepG2 Cells Treated with m-TOR Inhibitor Drugs at 37 °C in a 5% CO_2 incubator for 24 h. at a magnification of 20X. A) Control untreated HepG2 cells; B) HepG2 cells treated with sirolimus. C) HepG2 cells treated with everolimus; D) HepG2 cells treated with tacrolimus. Scale bar 100 µm.

activated caspases, although their effects were less pronounced than with everolimus. These inhibitors caused 0.2 to 0.3-fold increases for each caspase ($p < 0.05$), as reflected in Figures 2B, 2C, and 2D.

m-TOR Inhibitors induce morphological changes and cell death in HepG2 Cells

Microscopic evaluation of HepG2 cells following 48-hour treatment with m-TOR inhibitors (sirolimus, everolimus, and tacrolimus) at their respective IC_{50} concentrations revealed pronounced morphological changes indicative of cell death and apoptosis. Compared to untreated controls, treated cells exhibited increased cellular shrinkage and accumulation of dead cells and apoptotic bodies (Figure 3). Notably, everolimus treatment appeared to induce the most significant morphological alterations, exceeding the effects of sirolimus and tacrolimus.

m-TOR Inhibitor drugs Induce Cell Cycle Arrest in HepG2 Cells

The Cytell™ cell imaging system revealed profound cell cycle arrest in HepG2 cells upon 48-hour treatment with m-TOR inhibitors at their respective IC_{50} concentrations.

Effects on Different Phases as follows

Everolimus, Caused the most significant arrest, inducing substantial accumulation ($p < 0.001$) in G0/G1 (48.6%), S (8.73%), and G2/M (4.17%) phases compared to controls (Figure 4A). Tacrolimus, Induced significant accumulation ($p < 0.01$) in G0/G1 (37.4%) and S (6.17%) phases, with a moderate increase ($p < 0.05$) in G2/M (2%) (Figure 4A).

Sirolimus

Led to a noticeable, but less pronounced, arrest, with significant accumulation ($p < 0.05$) only in G0/G1 (27.5%) and S (4.1%) phases (Figure 4A). Notably, G2/M phase

Table 2. Docking of m-TOR Inhibitor Drugs, Specifically Sirolimus, Everolimus, Tacrolimus, and Sovaldi, with Nonstructural Protein 5A (NS5A) of Hepatitis C virus, NS5B RNA-dependent RNA, and Human protein kinase N2 (PKN2, PRKCL2)

Protein	Ligands	Docking	No. of H-Bonds	No. of H-Bonds	Pi-pi interaction
NS5A PDB:6P6Z	Everolimus	-3.505	1	Lys 1136	
	Sirolimus	-2.568		Gln 1041, Val 1078	
	Sovaldi	-4.789	4	Gln 1041, Ser 1042, Hip 1057, and Gly 1137	Hip 1057
	Tacrolimus	-3.735	4	Lys 1136	None
NS5B PDB: 4KHM	Everolimus	-1.983	0	None	None
	Sirolimus	-1.958	0	None	None
	Sovaldi	-4.647	3	Asp 352, Arg 355, and Glu 357	None
	Tacrolimus	-4.112	1	Asp 352	None
PKN2 PDB: 4CRS	Everolimus	-3.69	2	Asp 786, and Glu 822	None
	Sirolimus	-3.002	1	Asp 786	None
	Sovaldi	-5.377	2	Glu 705, and Phe 668	Arg 696, and Lys 684
	Tacrolimus	-3.695	0	-	-

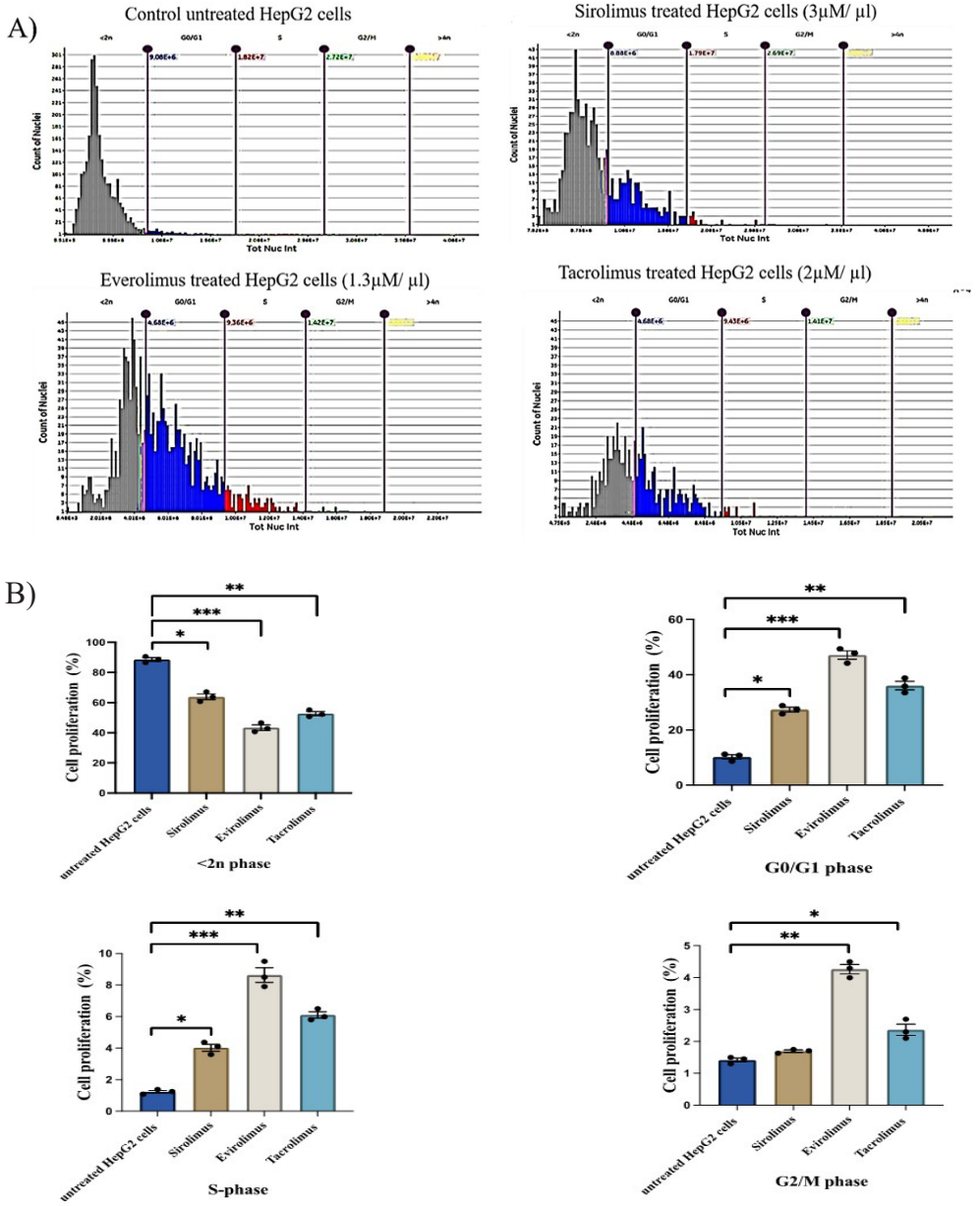

Figure 4. A) Cytell™ Cell Imaging System Charts of Cell Cycle Analysis of HepG2 Cells after Treatment with m-TORs Inhibitors Drugs at IC_{50} Concentration for 48 hrs. B) Quantification of cell cycle phases after treatment of HepG2 cells by m-TORs inhibitors drugs which represent <2n, G0/G1, S, and G2/M phases. The mean of three independent replicates was used to calculate these data values. Data presented as mean ± SD. (*) represents p-value < 0.05 and (**) represents p-value < 0.01, (***) represents p-value < 0.001 compared to control untreated HepG2.

remained unchanged compared to controls. All three inhibitors significantly decreased the <2n cell population compared to untreated cells (86.4%). Specifically, everolimus showed the strongest effect (38.5%, $p < 0.001$), followed by tacrolimus (54.8%, $p < 0.01$) and sirolimus (66.6%, $p < 0.05$) (Figure 4B).

m-TOR Inhibitors Suppress HCV Viral Load in Infected PBMCs

Real-time PCR analysis revealed a substantial reduction in HCV viral load following treatment of infected PBMCs with m-TOR inhibitors at their respective IC_{50} concentrations for 48 hours. The initial viral titer of 1.5 x 10^6 IU/mL was significantly decreased to around 8.71 x 10^5 IU/mL after infection with HCV serum. Notably, treatment with each m-TOR inhibitor further reduced the viral titer compared to infected, untreated PBMCs (Figure 5): Sirolimus, achieved the most significant reduction ($p < 0.001$) with a viral titer of around 4.91 x 10^5 IU/mL. Everolimus led to a significant decrease ($p < 0.01$) with a viral titer of around 6.08 x 10^5 IU/mL. Tacrolimus, showed a moderate but significant reduction ($p < 0.05$) with a viral titer of around 7.13 x 10^5 IU/mL.

These findings suggest that m-TOR inhibitors hold promise as potential therapeutic agents for controlling HCV infection, particularly sirolimus due to their superior antiviral activity in this study.

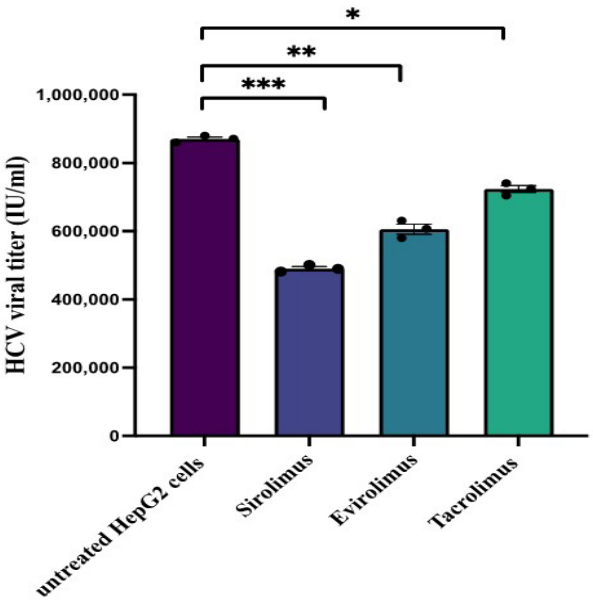

Figure 5. Results of Real-Time PCR of Infected Lymphocyte Cells Treated with m-TOR Inhibitor Drugs (Sirolimus, Everolimus, and Tacrolimus at IC_{50} concentrations for 48 h. The mean of three independent replicates was used to calculate these data values. Data presented as mean±SD. (*) represents p-value < 0.05 and (**) represents p-value < 0.01, (***) represents p-value < 0.001 compared to control untreated infected PBMC cells.

m-TOR Inhibitors Impede HCV Colony Formation in Infected PBMCs

Colony formation assays revealed that m-TOR inhibitor treatment significantly reduced HCV colony formation in infected PBMCs compared to untreated controls (Figure 6). Notably, sirolimus demonstrated the most pronounced effect, leading to a greater decrease in colony formation compared to both everolimus and tacrolimus.

m-TOR Inhibitors Suppress TNF-α Production in HCV-Infected PBMCs

ELISA analysis revealed that m-TOR inhibitor treatment significantly reduced the production of tumor necrosis factor-α (TNF-α) in HCV-infected PBMCs compared to untreated controls (Figure 7). Sirolimus treatment triggered the most substantial decrease ($p < 0.01$), with a remarkable 180.6-fold reduction in TNF-α activity compared to untreated cells. Everolimus and tacrolimus also significantly suppressed TNF-α production ($p < 0.05$), achieving reductions of 65.84 and 35.2 folds, respectively. These findings suggest that m-TOR inhibitors, particularly sirolimus, hold promise for mitigating inflammatory responses associated with HCV infection by modulating TNF-α production.

m-TOR inhibitors drugs have inhibitory activity against Human protein kinase N2 (PKN2), NS5A and NSB5 in silico

Molecular docking designated using the glide program software to explore the binding of m-TOR inhibitor drugs, Sirolimus, Everolimus, Tacrolimus, and Sovaldi, to the active sites of NS5A, NS5B and (PKN2). The findings indicated that these drugs could bind to the ligand-binding domain of NS5A, NS5B and PKN2, leading to a reduction in their activity.

As for NS5A, Sovaldi had a binding energy of -4.789 kcal/mol, with the formation of four hydrogen bonds with Gln 1041, Ser 1042, Hip 1057, and Gly 1137. Additionally, Sovaldi also formed a pi-pi interaction with Hip 1057. This suggests that Sovaldi may be the most potent inhibitor of NS5A studied. Tacrolimus possessed a binding force of -3.735 kcal/mol, with the formation of four hydrogen bonds with Ser 1139, Gly 1137, Leu 1135, and Ala 1157. This suggests that Tacrolimus may also be a potent inhibitor of NS5A. Everolimus had binding energy of -3.505 kcal/mol, with the formation of a single hydrogen bond with Lys 1136. This suggests that Everolimus may

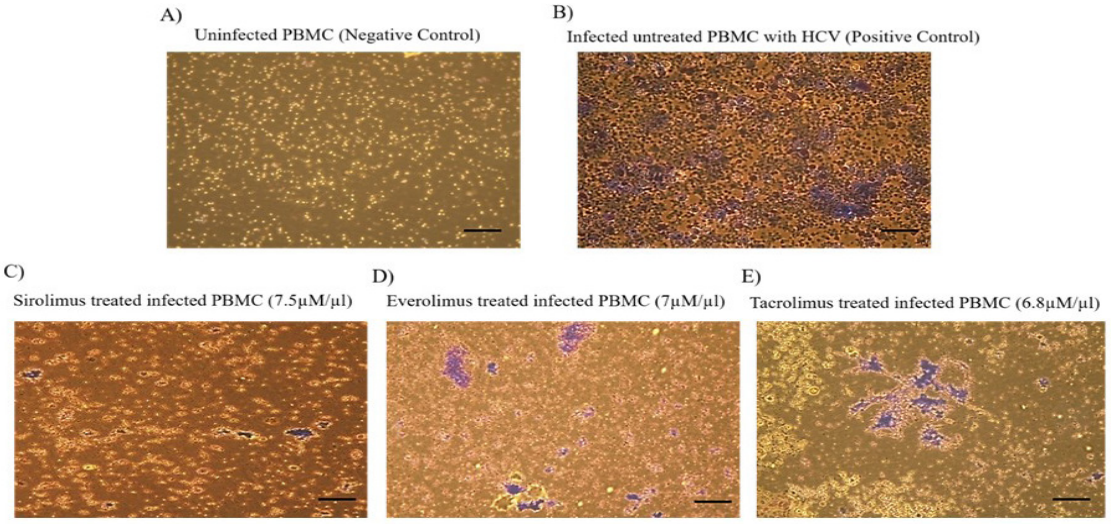

Figure 6. Effect of m-TOR Inhibitor Drugs at IC_{50} Concentrations for 48h on Colony Formation of PBMC Cells that were Infected with 1 ml (1.5×10^6 HCV) serum. A) Uninfected PBMC cells with HCV serum (Negative control), B) untreated infected PBMC cells with HCV (positive control), C) Sirolimus treated infected PBMC cells at IC_{50} concentration (7.5µM/µl), D) Everolimus treated infected PBMC cells at IC_{50} concentration (7 µM/µl), E) Tacrolimus treated infected PBMC cells at IC_{50} concentration (6.8 µM/µl). Scale bar 7 µm at magnification 40 X.

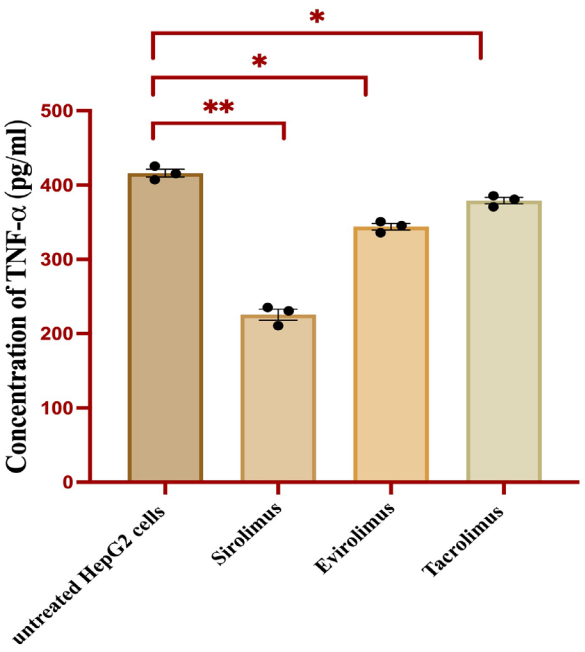

Figure 7. Quantification of the Changes in the Activity of TNF-α in Infected PBMC Cells with HCV Serum Following Treatment with Sirolimus, Everolimus, and Tacrolimus at IC_{50} Concentration for 48 hrs. The mean of three independent replicates was used to calculate these data values. Data presented as mean±SD. (*) represents p-value < 0.05 and (**) represents p-value < 0.01 compared to control untreated infected PBMC cells.

be a more potent inhibitor of NS5A than the other ligands studied. Sirolimus showed the lowest binding energy of -2.568 kcal/mol, with the formation of two hydrogen bonds with Gln 1041 and Val 1078. This suggests that Sirolimus may also be a potent inhibitor of NS5A as shown in Table 2 and Figures 8A, 8B, 8C, and 8C.

As for NS5B, Also Sovaldi exhibited a high docking score of -4.647 kcal/mol, indicating a strong binding affinity to NS5B. Sovaldi formed three hydrogen bonds with important residues including Asp 352, Arg 355, and Glu 357, which potentially contribute to the strong binding between Sovaldi and NS5B. NS5B showed strong affinity towards Tacrolimus with a docking score of -4.112 kcal/mol and a specific interaction with Asp 352 through a hydrogen bond. On the other hand, the docking scores for Sirolimus and Everolimus were -1.958 kcal/mol and -1.983 kcal/mol, respectively, indicating moderate affinities towards NS5B without any hydrogen bonds formed, resulting in a lack of strong interactions with the target protein as shown in Table 2 and Figures 9A, 9B, 9C, and 9C.

The docking study results provided insights into the interactions between the ligands and PKN2 as well. Sovaldi had the highest binding affinity, with -5.377 kcal/mol for a docking score, and formed two important hydrogen bonds with Glu 705 and Phe 668, indicating specific interactions required for binding. Additionally, Pi-pi interactions with Arg 696 and Lys 684 contributed to the stability of the

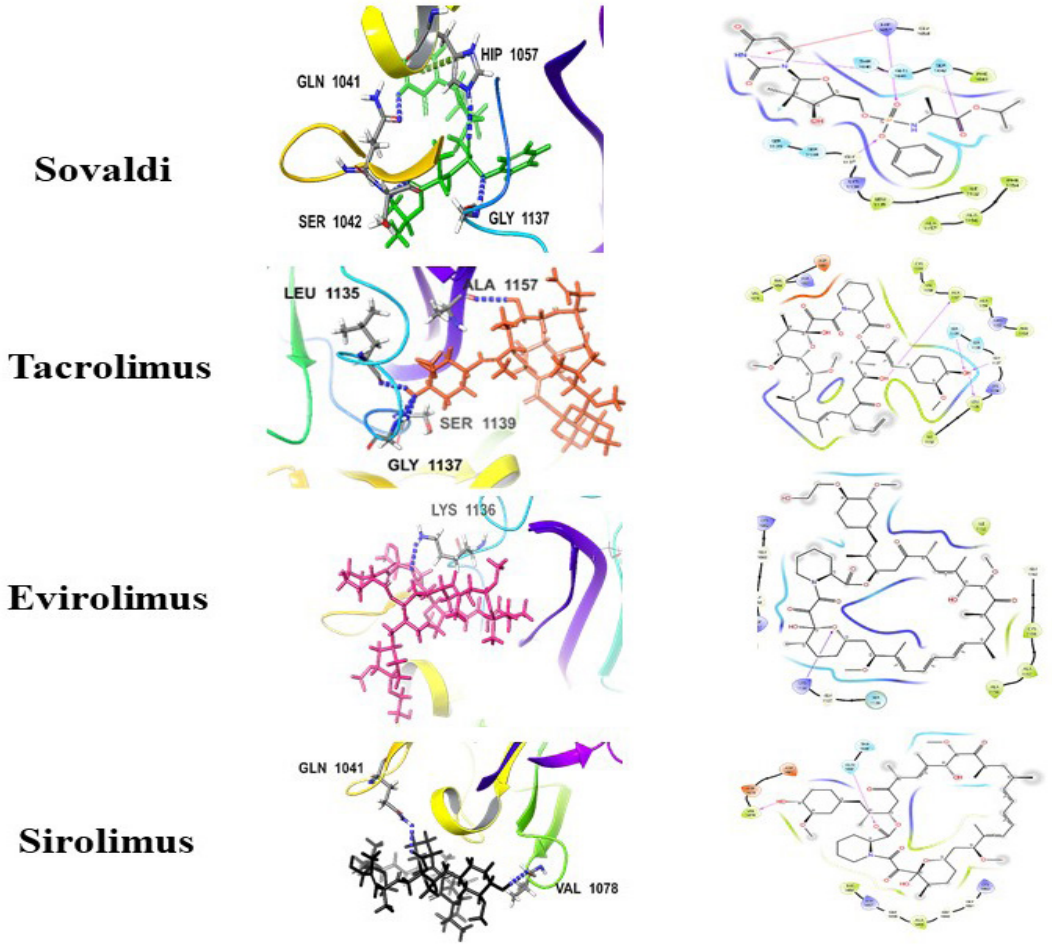

Figure 8. Binding Modes of Sovaldi and m-TORs Inhibitors Drugs with Nonstructural Protein 5A (NS5A)

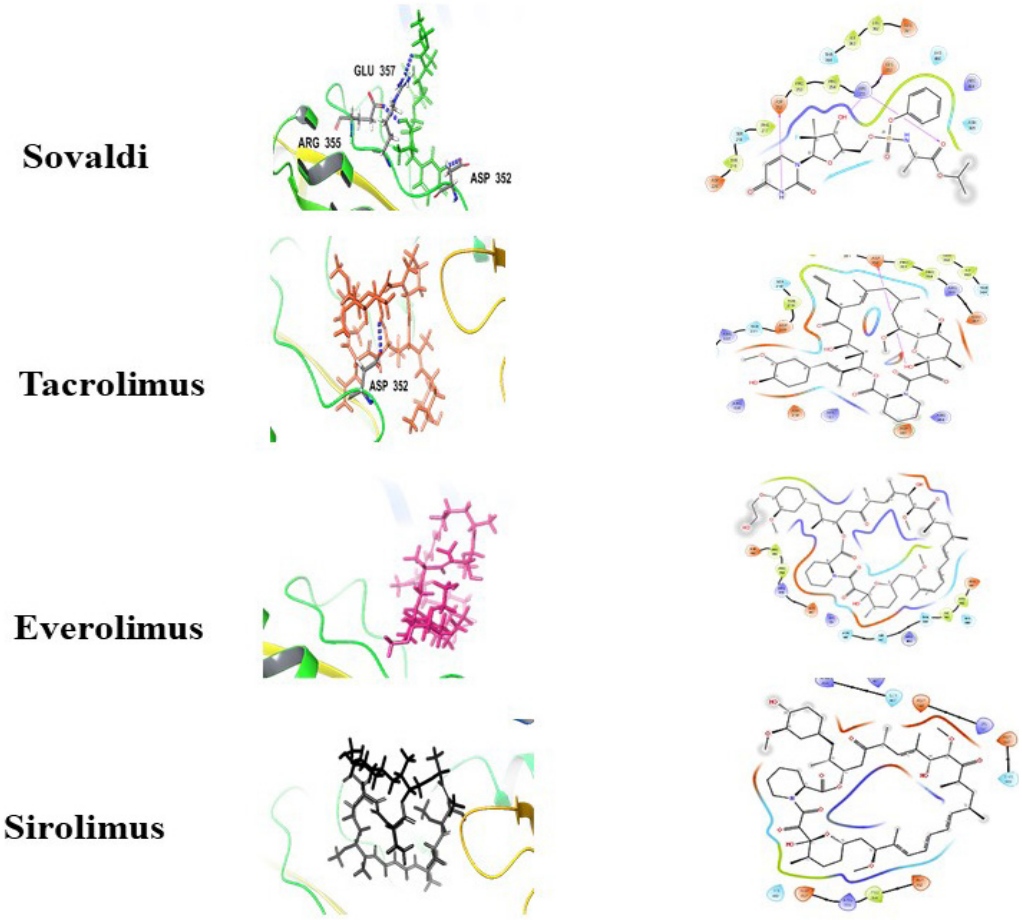

Figure 9. Binding Modes of Sovaldi and m-TORs Inhibitors Drugs with NS5B

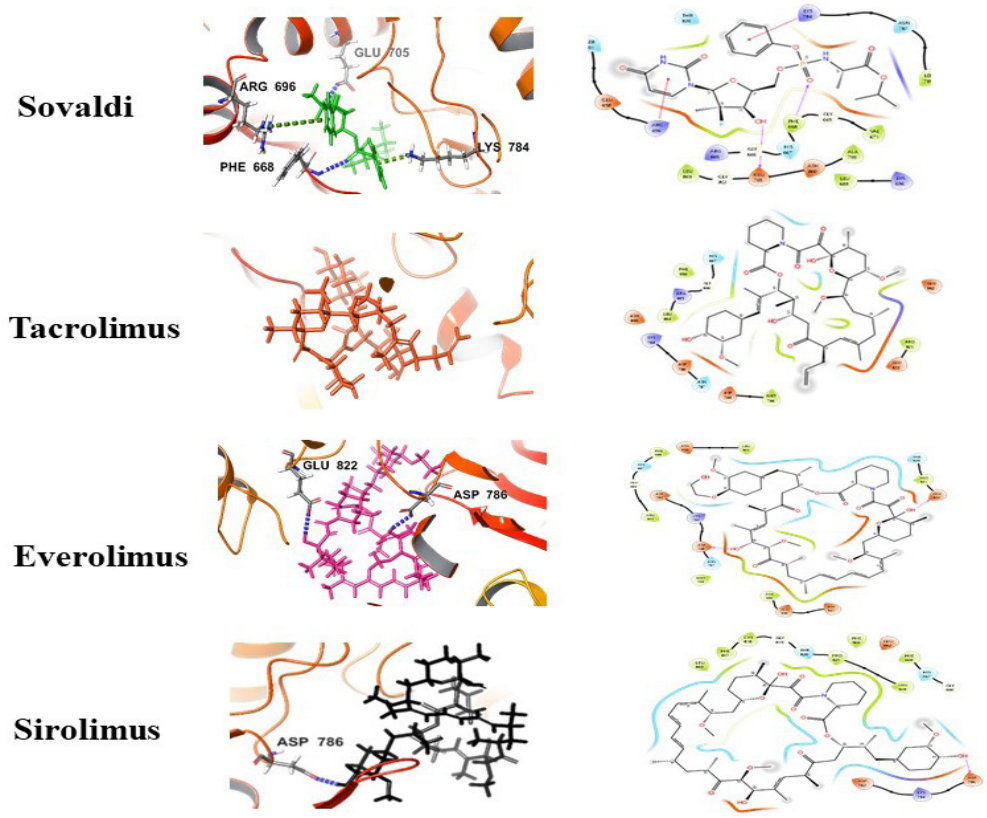

Figure 10. Binding Modes of Sovaldi and m-TORs Inhibitors Drugs with Human Protein Kinase N2 (PKN2)

complex. Tacrolimus displayed a weaker binding affinity, with -3.695 kcal/mol for the docking, and did not form any hydrogen bonds. However, it showed potential binding affinity towards PKN2. Everolimus had a moderate binding affinity, with a docking score of -3.690 kcal/mol, and formed two hydrogen bonds with Asp 786 and Glu 822 at the binding site. Sirolimus had a relatively weaker binding affinity, with a slightly lower docking score of -3.002 kcal/mol, and formed a single hydrogen bond with Asp 786, suggesting potential interaction with PKN2 as shown in Table 2 and Figures 10A, 10B, 10C, and 10D.

Discussion

Various signaling pathways that influence invasion, metastasis, cell proliferation and angiogenesis are de-regulated in Hepatocellular carcinoma (HCC) [29]. Deregulation of m-TOR pathway is essential to the growth and progression of HCC. In addition, m-TOR pathway is a target for HCV nonstructural protein 5A (NS5A), an essential component of several cellular processes and viral replication [10]. This present study investigated the role of m-TOR inhibitor drugs (sirolimus, everolimus and tacrolimus) as anti-cancer against a HCC cell line HepG2, and also their role as anti-viral against HCV. We determined that the HepG2 cells' cell survival test post-treatment with m-TOR inhibitor drugs (sirolimus, everolimus and tacrolimus) revealed a significant decrease in cell viability at concentrations of 5µM/ µl and 10 µM / µl while it wasn`t affected after at concentration 1 µM /µl. Previous research shows a significant decline in HepG2 cell viability supports by 15 % and 23% after treatment with rapamycin at concentrations of 40 mM and 50 mM respectively for 72 hrs [30]. A different study revealed that everolimus induced growth inhibition by 90-95 % in different HCC cell lines including Hep3B, HepG2, PLC/PRF/5, and SNU398 at a concentration of 20 µM for 48 hrs [6]. Moreover, tacrolimus reduced cell viability and growth frequency in Huh7 and HepG2 cells by 50%, and IC_{50} was reached to 0.07µM and 0.047 µM respectively after 48 hrs [31]. On the contrary, we found that treatment of PBMC with m-TOR inhibitors (sirolimus, everolimus and tacrolimus) has no synergetic effect on the cell viability of PBMC. Our findings concur with those of an earlier investigation, which revealed that rapamycin the yield and proliferation of Vγ2Vδ2 cells in PBMC [32]. Another study showed that rapamycin increases the cytotoxic effect and decreases cell viability of leukemia cells, but protects and stimulates the survival of normal lymphocytes [33].

The current study looked into the antioxidant effect of m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus), where they have caused a marked increase in total antioxidant, glutathione S-transferase, and glutathione reductase levels in HepG2 cells compared to control untreated HepG2 cells. A previous study reported that rapamycin increases the oxidation status by elevating the level of GSH and total antioxidant capacity, protein oxidation and lipid peroxidation in MDA-MB-231 and MCF-7 cell lines compared to control cells [34]. Another study showed that rapamycin in combination with fructose-1,6-bisphosphate (FBP) can enhance oxidative stress in HepG2 cells by increasing thiobarbituric acid reactive substances (TBARS) [30].

The finding of the current study indicated that m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) possessed high apoptotic effect against HepG2 cells, where they significantly increased the apoptotic markers including caspase (3, 8, and 9) and fas-ligand compared to untreated HepG2 cells. The above results agreed with previous studies which indicated that rapamycin triggers apoptosis by increasing caspase-3 level, decreasing Bcl-2 expression, and inducing the expression of pro-apoptotic marker Bcl-XL [35, 22]. Another study showed that everolimus induces apoptosis in neuroendocrine tumors by triggering caspase-3 expression and decreasing KI-67 expression [36]. A different study reported that tacrolimus increases human leukemia apoptosis Jurkat cells by the activation of the 3, 8, and 9 caspases [37]. The results of the phenotypic analysis of liver cancer cells showed treatment with m-TOR inhibitor drugs had a significant inhibitory effect on the growth of cancer cells, which led to an increase in dead cells in addition to the presence of shrinkage signals and apoptosis compared to untreated HepG2 cells. Previous studies supported our results, for example; rapamycin increases apoptotic hallmarks such as cell shrinkage and MCF-7 treated cells vascularization compared to untreated cells (control cells) [21].

Our results revealed that treatment with an m-TOR inhibitor had an inhibitory effect and stopped the cycle of hepatocellular carcinoma (HepG2) in both the G0/G1 phase and the G2/M phase and a similar decrease in the <2n phase. It is also possible for the cell cycle to stop before completing the replication process, and drugs at IC_{50} concentrations compared to control untreated HepG2 cells. These findings in accordance with a previous study which reported that treatment of MCF-7 with rapamycin causes a growth in the number of G0G1 phase cells and a corresponding decline in the synthesis (s) and mitosis (G2M) phases populations [21]. Another previous study showed that a high concentration of tacrolimus and everolimus (100µM) induces accumulation of HepG2 cells in G0/G1 phase, but sirolimus induces accumulation of Hepatocellular carcinoma (HepG2) cells in G2+M phases compared to untreated control cells [20]. The effect of m-TOR inhibitor drugs (sirolimus, everolimus and tacrolimus) on HCV replication indicated a decrease in the viral titer in the PBMC infected after treatment with m-TOR inhibitor drugs at IC_{50} concentrations for 48 h; viral load greatly reduced after treating with sirolimus, everolimus and tacrolimus.

The effect of m-TOR inhibitor drugs on hepatitis C virus viral replication and viral colony formation was tested compared to the control group (cells not inoculated with the virus). The results found that the effect of m-TOR inhibitor drugs significantly reduces colony formation and viral reproduction. In addition, our results of the effects of m-TOR inhibitor drugs on TNF-α in infected PBMC with HCV showed a marked reduction in TNF-α activity compared to untreated infected PBMC.

As of our last knowledge update, the specific effects of m-TOR pathway on HCV infection and replication

are complex and not fully understood. The interactions between viruses like HCV and host cellular pathways can be intricate, and research is ongoing to uncover the details of these interactions. However, according previous studies and research [9-12], there is a relation between m-TOR and HCV replication through NS5A, NS5B and PKN2, which support us in conducting in-silico docking studies between m-TOR inhibitor drugs (sirolimus, everolimus, and tacrolimus) and sovaldi as indeed an important and widely used medication for HCV infection treatment with NS5A, NS5B and PKN2.Based on the results, it is reported that there is a good inhibitory activity and binding affinity of sirolimus against NS5B and PKN2 compared to everolimus, tacrolimus, and sovaldi.

Our results agreed with a previous study which demonstrated that sirolimus is efficient in reducing viral load and recurrence in HCV patients after liver transplantation [38]. Another result reported that everolimus effects on HCV replication in genotype (GT) 1b, GTb2, and GTb3 by overexpression of promyelocytic leukemia (PML) by tumor suppressor protein has role in viral response and apoptosis [39-41]. The in-silico study's findings indicate that each of the four ligands has the potential to interact and inhibit the functionality of NS5A. However, Sovaldi shows the highest binding affinity [42], followed by Everolimus, Tacrolimus, and Sirolimus. The docked results indicate that all four ligands can obstruct NS5A, with the most significant inhibitory effect seen in Sovaldi, followed by Everolimus, Sirolimus, and Tacrolimus. In addition to binding energy, the docking studies revealed other important aspects of the ligand-NS5A interactions. The amino acid Lys 1136 may be a potential target for the development of new NS5A inhibitors, as Everolimus, Sirolimus, and Sovaldi were found to form hydrogen bonds with them. Moreover, a pi-pi interaction with Hip 1057 was observed for Sovaldi, suggesting its potential contribution to NS5A inhibition.

In conclusion, in our current study, the effects of some antioxidants were tested along with the ability of m-TOR inhibitory drugs on HepG2 liver cancer cells. In addition, the ability of these substances to inhibit viral growth in PBMC cells infected with hepatitis C virus was tested. Finally, we would like to point out that it is possible in the future to investigate the ability of these substances as anti-hepatitis C viruses (HCV) and anti-cancer agents such as liver cancer (HCC).

Author Contribution Statement

Literature search: D.M, W.A, and S.S.; Lab work and methodology design: D.M.; data curation and analysis: D.M, M.R, and A.H.I.F.; original draft preparation, D.M.; writing review and editing D.M., R.N.M, A.H.I.F and S.S.; visualization: D.M., A.H.I.F and S.S.; supervision: W.A. and S.S. All authors have read and agreed to the published version of the manuscript.

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Data availability

All data that were used and analyzed in this study have been included for publication.

Conflicting interests

The authors declare no conflicts of interest.

Ethics clearance and participation consent

The study was approved by the Ethics Committee of the National Cancer Institute, Cairo University, Cairo, Egypt (IRB No. 00004025, IORG. 0003381). Informed consent statement: All patients gave informed consent.

Agreement to Publication

The corresponding author was authorized by the co-authors to approve the publication of the manuscript.

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