Original Article

Targeting PAR-2-driven inflammatory pathways in colorectal cancer: mechanistic insights from atorvastatin and rosuvastatin treatment in cell line models

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Background: Colorectal cancer (CRC) is a growing health concern globally and in regions such as the United Arab Emirates, where risk factors like obesity and hyperlipidaemia are prevalent. Chronic inflammation, driven by pathways involving protease-activated receptor 2 (PAR-2), plays a pivotal role in CRC progression, creating a tumour-promoting microenvironment. The overexpression of PAR-2 has been associated with increased tumour aggressiveness and drug resistance. While previous studies have focused on broad inflammatory modulation, this study explores the selective targeting of PAR-2 by atorvastatin (ATV) and rosuvastatin (RSV), highlighting their specificity by assessing minimal impact on PAR-1 expression, which serves as a control.

Methods: HT-29 and Caco-2 CRC cell lines were employed to investigate the anti-inflammatory effects of ATV and RSV. Inflammation was induced with lipopolysaccharide (LPS), followed by treatment with varying concentrations of ATV and RSV. Western blotting and real-time polymerase chain reaction for quantification (qPCR) were performed to quantify PAR-2 and TNF-α at both the protein and mRNA levels. Enzyme linked immunosorbent assay (ELISA) was used to measure the secretion of TNF-α. Calcium signalling, which plays a crucial role in inflammation, was analysed using Fluo-4 AM dye, with fluorescence imaging capturing the effects of statin treatment on intracellular calcium influx.

Results: LPS treatment significantly upregulated PAR-2 and TNF-α expression in both cell lines, validating the inflammatory model. Co-treatment with ATV or RSV reduced PAR-2 and TNF-α expression in a dose-dependent manner. The higher concentrations of ATV (50 µg/mL) and RSV (20 µg/mL) produced the most significant reduction in these inflammatory markers at both the protein and mRNA levels. Importantly, the treatment did not substantially alter PAR-1 expression, underlining the specificity of ATV and RSV in modulating PAR-2-mediated pathways. Additionally, statin treatment attenuated LPS-induced calcium influx, with fluorescence intensity decreasing markedly at higher concentrations of both statins.

Conclusions: This study provides novel insights into the selective targeting of PAR-2 by ATV and RSV, distinguishing their effects from PAR-1. The reduction in PAR-2 expression and TNF-α secretion, along with the suppression of calcium signalling, underscores the potential of these statins as targeted anti-inflammatory agents in CRC. The findings highlight the therapeutic value of ATV and RSV in modulating

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inflammation through PAR-2-specific pathways, which may contribute to reduced cancer progression. These results pave the way for further preclinical and clinical evaluations to explore statins as adjunctive therapies in the management of CRC.

Keywords: Protease-activated receptor 2 (PAR-2); statins; colorectal cancer (CRC); inflammation; calcium signalling

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Introduction

Background

Colorectal cancer (CRC) remains a formidable global health challenge, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality worldwide (1). In the United Arab Emirates (UAE), where obesity and hyperlipidemia have seen a marked increase (2), CRC holds significant epidemiological relevance, standing as the second most prevalent cancer in males and third in females (3,4). The UAE also ranks among the top 10 countries globally for obesity, with over 30% of the adult population classified as obese (5). This rise in obesity, a key risk factor for CRC, further amplifies the burden on the healthcare system, as both obesity and hyperlipidemia contribute to CRC progression (6-9).

These conditions drive chronic, low-grade inflammation, which contributes to tumor initiation and progression through the disruption of adipose tissue homeostasis and altered lipid metabolism (8,10,11). This pro-inflammatory state fosters a microenvironment conducive to oncogenesis, underscoring the importance of targeting inflammation in CRC prevention and therapeutic strategies.

Chronic inflammation is also strongly implicated in CRC pathogenesis, with inflammatory conditions such as inflammatory bowel disease (IBD), ulcerative colitis (UC), and Crohn's disease significantly increasing the risk for CRC (12,13). Inflammation-mediated epithelial damage leads to genetic mutations and dysregulated signaling that promote tumor initiation and progression. For example, chronic inflammation in conditions like UC can induce oxidative stress, resulting in mutations of the *TP53* gene, a crucial tumor suppressor, which drives CRC progression (14). Additionally, in Crohn's disease, the inflammatory microenvironment is linked to Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations, promoting unchecked cell proliferation and survival, further facilitating tumorigenesis and the progression of CRC (15). Additionally, obesity and hyperlipidemia exacerbate CRC outcomes by inducing insulin resistance, oxidative stress, and dysregulated cytokine signaling, further intensifying the inflammatory state and accelerating disease progression (16). Insulin resistance has been identified as a significant risk

Highlight box

Key findings
- This study demonstrates that atorvastatin (ATV) and rosuvastatin (RSV) selectively inhibit protease-activated receptor-2 (PAR-2) expression and tumour necrosis factor-α (TNF-α) secretion in lipopolysaccharide (LPS)-stimulated colorectal cancer (CRC) cell lines, HT-29 and Caco-2.
- Statin treatment also reduces calcium influx, highlighting the modulation of calcium-dependent inflammatory pathways.
- The expression of PAR-1 remains largely unaffected, emphasizing the specificity of ATV and RSV towards PAR-2.

What is known and what is new?
- PAR-2 overexpression is known to promote inflammation, tumour progression, and drug resistance in CRC, presenting challenges for therapeutic management.
- Statins are established as anti-inflammatory agents, but their specific impact on PAR-2 signaling in CRC models has not been thoroughly investigated.
- This study provides novel evidence of selective PAR-2 inhibition by ATV and RSV, with minimal effect on PAR-1 expression, along with calcium signalling suppression as a mechanism for reducing inflammation.

What is the implication, and what should change now?
- The findings highlight the potential of ATV and RSV as adjunctive therapies in CRC, offering targeted modulation of PAR-2-mediated inflammatory pathways.
- This selective targeting could improve treatment outcomes by reducing inflammation-driven cancer progression while minimizing off-target effects on PAR-1.
- Further preclinical and clinical trials are needed to validate these findings and explore the integration of statins into CRC treatment regimens for enhanced therapeutic efficacy.

factor for CRC. A comprehensive cohort study highlighted that individuals with metabolic syndrome, a condition intrinsically linked to insulin resistance, exhibited a 33% elevated risk of developing CRC, underscoring the interplay between metabolic dysregulation and cancer progression (17). Another study found that hyperinsulinemia promotes the growth of colorectal tumors by increasing levels of insulin-like growth factor-1 (IGF-1), which enhances cell proliferation and survival (18).

Recent advances underscore the critical role of protease-activated receptor 2 (PAR-2) in mediating inflammation and its direct involvement in oncogenesis, particularly in the context of CRC (19). PAR-2, a member of the G-protein-coupled receptor (GPCR) family, is activated through proteolytic cleavage by specific serine proteases such as trypsin, tryptase, and coagulation factors like Factor Xa (20). Unlike other GPCRs, PAR-2 does not rely on traditional ligands but utilizes its tethered ligand mechanism for receptor activation, which facilitates its persistent involvement in inflammatory and proliferative processes (21). PAR-2 expression is significantly elevated in CRC compared to other malignancies such as breast, ovarian, and pancreatic cancers. A comparative analysis revealed that CRC tissues exhibit the highest expression of PAR-2 among these cancer types, correlating with more aggressive tumour behaviour and poorer prognosis (22). Even, expression of the PAR-2 gene (F2RL1) is significantly higher in CRC tissues compared to normal colonic tissues (22,23). This elevated expression of PAR-2 in CRC underscores its pivotal role as a key mediator in the disease's pathogenesis, driving both inflammatory and proliferative processes. Not only does high PAR-2 expression correlate with a more aggressive clinical course, but it is also associated with poor prognosis in CRC patients (24). This makes PAR-2 a compelling prognostic marker and a viable therapeutic target, offering potential pathways for interventions aimed at mitigating inflammation-driven tumor progression and improving clinical outcomes.

Functionally, PAR-2 promotes several malignant phenotypes in CRC, including tumor cell proliferation, migration, invasion, and metastasis (25). It enhances tumour progression by activating key oncogenic pathways, particularly the β-catenin and periostin pathways, which drive cancer stem cell (CSC) self-renewal and metastatic dissemination (26). Furthermore, PAR-2 contributes to the epithelial-mesenchymal transition (EMT), a crucial mechanism underlying cancer invasion and metastasis (27). PAR-2's role in EMT underscores its importance in enhancing the invasive capabilities of CRC cells and promoting metastatic spread.

Recent discoveries have also identified the PAR-2-LRP6-Axin and PAR-2-RNF43-β-Catenin pathways as key modulators of CRC progression. The PAR-2-LRP6-Axin signaling axis plays a pivotal role in stabilizing β-catenin, thereby preventing its degradation and promoting oncogenesis in CRC. Upon activation, PAR-2 initiates downstream signaling that recruits LRP6 (28). Phosphorylation of LRP6 at PPPS/TP motifs by Casein Kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β) is essential for its function. Once phosphorylated, LRP6 interacts with Axin, a scaffold protein in the β-catenin destruction complex, preventing Axin from facilitating β-catenin degradation (29). Normally, the 'destruction complex', which includes adenomatous polyposis coli (APC), CK1, and GSK-3β, phosphorylates β-catenin, leading to its ubiquitination and proteasomal degradation (30). However, the LRP6-Axin interaction disrupts this process, resulting in β-catenin stabilization. Stabilized β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it forms a complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, driving the transcription of oncogenic genes such as *c-Myc* and *Cyclin D1* (31). *c-Myc* promotes cell cycle progression and proliferation (32), while *Cyclin D1* facilitates the G1 to S phase transition (33), contributing to tumour growth. This signalling axis also intersects with the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways, enhancing cell survival and invasion (34). Furthermore, it plays a critical role in maintaining CSCs, which drive tumour initiation and resistance to therapy (35). Through EMT, the PAR-2-LRP6-Axin signalling axis further contributes to cancer progression and metastasis. This process involves the activation of key transcription factors, including *Snail* and *Twist*, which are known to enhance cell motility and invasiveness (28). The interaction between PAR-2 and LRP6 promotes rapid phosphorylation of LRP6, resulting in the recruitment of Axin from the "destruction-complex" pool, as indicated above (28). This recruitment not only stabilizes β-catenin but also facilitates the formation of LRP6 signalosomes, which include Frizzled (FZD), Axin, and GSK3 (36). The formation of these signalosomes is facilitated by the production of phosphatidylinositol 4,5-bisphosphate (PIP2), synthesized by phosphatidylinositol-4-phosphate 5-kinase type-1 (PIP5K1) when bound to Dishevelled (DVL) (36). This

interaction accelerates PIP2 generation, which enhances and amplifies Wnt/β-catenin signalling. In the context of CRC, Wnt-signaling plays a pivotal role in driving tumorigenesis. The aberrant activation of this pathway, commonly through mutations in APC or β-catenin, leads to the stabilization and nuclear translocation of β-catenin. This results in the transcription of genes involved in cell proliferation, survival, and metastasis, contributing to the initiation and progression of CRC. The amplification of Wnt signaling through PIP2 production further exacerbates these oncogenic processes (37).

Moreover, the PAR-2-LRP6-Axin axis interacts with multiple other signalling pathways. For instance, the (Pro) renin receptor (PRR) has been identified as a component of the Wnt receptor complex that can promote and augment Wnt signaling (38). PRR acts as a specific adaptor between LRP6 and V-ATPase, which is essential for LRP6 phosphorylation and activation (38). Also, insulin-like growth factor binding protein 2 (IGFBP2) has been shown to interact with LRP6, enhancing β-catenin transcriptional activity (39). This interaction provides another layer of regulation to the PAR-2-LRP6-Axin signaling axis. The complex interplay between these various components underscores the intricate regulation of the PAR-2-LRP6-Axin signaling axis and its far-reaching effects on cancer progression, metastasis, and therapy resistance.

The PAR-2-RNF43-β-Catenin pathway is another crucial signalling mechanism in CRC that modulates β-catenin stability and promotes tumorigenesis (40). While the PAR-2-LRP6-Axin axis involves β-catenin stabilization through Axin sequestration, the PAR-2-RNF43 pathway focuses on regulating FZD receptors, key components in the Wnt/β-catenin signalling pathway (40). RNF43 (Ring Finger 43), an E3 ubiquitin ligase, typically functions to ubiquitinate and degrade FZD receptors, limiting Wnt signalling (41). However, upon activation of PAR-2, RNF43's function is inhibited, allowing an accumulation of FZD receptors at the cell membrane, thus amplifying Wnt signalling and stabilizing β-catenin (40).

One notable example comes from a study conducted by Giannakis *et al.*, which identified RNF43 mutations in a significant subset of microsatellite instability-high (MSI-H) CRC patients (42). In these tumours, loss-of-function mutations in RNF43 led to the inactivation of its ubiquitin ligase activity, causing continuous FZD receptor activation and constitutive Wnt/β-catenin signalling (42). This mutation-driven hyperactivation of β-catenin contributes directly to tumour growth, metastasis, and resistance to standard therapies. The study highlighted that nearly 18% of CRC patients with MSI-H tumours harboured RNF43 mutations, underscoring the clinical relevance of this pathway in CRC progression (42).

In another case, Koo *et al.* demonstrated that RNF43 mutations in CRC promote CSC renewal by sustaining β-catenin-driven transcription of stemness-associated genes, such as *LGR5* and *ASCL2* (43). These mutations were particularly prevalent in right-sided CRCs, a subtype known for its distinct molecular features and poorer prognosis compared to left-sided tumours (43). The RNF43 mutation, by sustaining Wnt signaling, helps maintain a population of stem-like cells within tumours, facilitating both tumour initiation and resistance to chemotherapy. These exemplars illustrate the critical role that RNF43 mutations play in CRC pathogenesis, especially in terms of sustaining β-catenin activity and driving oncogenic processes. Hence, the PAR-2-RNF43-β-Catenin axis not only represents a key regulatory mechanism of Wnt signalling in CRC but also highlights the clinical importance of RNF43 as a biomarker and a therapeutic target.

In addition to the pathways mentioned above, PAR-2 plays a critical role in regulating several well-characterized cell signalling pathways. Activation of PAR-2 has been shown to stimulate the MEK1/2-ERK1/2 signaling cascade across various cell types. Additionally, PAR-2-induced ERK1/2 activation is mediated through both G protein-dependent and -independent mechanisms. The G protein-dependent pathway involves Gq/11 and Gi/o, leading to the activation of protein kinase C (PKC) and subsequent phosphorylation of ERK1/2, while the G protein-independent mechanism involves β-arrestin recruitment, acting as a scaffold for signalling complexes, including RAF, MEK, and ERK (44). In cancer cells, PAR-2-mediated ERK1/2 activation has been associated with increased cell proliferation, migration, and invasion. Furthermore, recent findings suggest that PAR-2-induced ERK1/2 activation can lead to the upregulation of matrix metalloproteinases (MMPs), particularly MMP-9, contributing to enhanced tumor metastasis (19).

PAR-2 has also been found to interact with TGF-β signalling in complex ways, where TGF-β plays a dual role in inflammation and CRC. It acts as an anti-inflammatory agent in normal tissue but promotes tumour progression and immune evasion in CRC by inducing EMT and enhancing tumour cell survival (45). This effect is mediated through the increased phosphorylation and nuclear translocation of Smad2/3, key components of the TGF-β

signaling pathway (46). Additionally, PAR-2 activation specifically through the MEK1/2-ERK1/2 pathway has been shown to elevate the expression of TGF-β1, creating a positive feedback loop that amplifies the tumour-promoting effects of both the PAR-2-MEK1/2-ERK1/2 and TGF-β/Smad2/3 pathways (47). This interaction between PAR-2 and TGF-β signaling has been implicated in promoting CSC-like properties and chemoresistance in several tumour types (48).

The interaction between PAR-2 and the PI3K/Akt pathway has gained significant attention in recent years. PAR-2 activation has been shown to rapidly induce PI3K activity, leading to increased phosphorylation and activation of Akt. This activation occurs through both G protein-dependent and -independent mechanisms, similar to the ERK1/2 pathway (44). Recent findings have revealed that PAR-2-mediated Akt activation plays a crucial role in promoting cancer cell survival and resistance to apoptosis. Furthermore, the PAR-2-PI3K/Akt axis has been implicated in the regulation of glucose metabolism in cancer cells, contributing to the Warburg effect and supporting tumor growth (49,50).

PAR-2 activation has been shown to stimulate NF-κB signaling, leading to increased expression of pro-inflammatory cytokines and chemokines (51). This mechanism contributes to the creation of a tumor-promoting inflammatory microenvironment. Emerging evidence suggests that PAR-2 may activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, particularly STAT3, in certain cancer types (52). This activation promotes cell survival, proliferation, and metastasis. Furthermore, recently PAR-2 activation has been shown to interact with the Hippo pathway, inhibiting its activity and resulting in increased nuclear localization of YAP/TAZ transcriptional coactivators, thereby promoting cancer cell proliferation and stemness (53).

In addition to driving tumor progression, as evidenced by its association with advanced clinicopathologic features such as depth of tumor invasion, liver metastasis, and tumor node metastasis (TNM) stage in human CRC, where 33.9% of 295 patients exhibited positive PAR-2 expression, and despite not being a significant negative prognostic factor for overall survival in either univariate or multivariate analyses (54), PAR-2 is implicated in mediating resistance to chemotherapeutic agents. For instance, PAR-2 activation has been shown to attenuate doxorubicin-induced cell death in colon cancer cells by upregulating anti-apoptotic proteins such as MCL-1 and Bcl-xL, thereby promoting cellular survival under chemotherapeutic stress (55). Moreover, PAR-2 inhibition has demonstrated the potential to sensitize CRC cells to EGFR-targeted therapies, enhancing the efficacy of combination treatments (56). These findings suggest that PAR-2 plays a crucial role in maintaining drug resistance, and its inhibition may reverse resistance mechanisms, particularly to chemotherapeutic agents like 5-fluorouracil (5-FU) and doxorubicin (57).

Given the robust association between PAR-2 expression and CRC progression, inhibiting this receptor has emerged as a promising therapeutic strategy. PAR-2 antagonism has demonstrated the potential to enhance the efficacy of standard chemotherapeutic agents, such as doxorubicin and 5-FU, both *in vitro* and *in vivo*, by disrupting critical survival and EMT pathways (58). Furthermore, PAR-2 inhibition has been shown to sensitize CRC cells to 5-FU, with siRNA-mediated depletion or pharmacologic inhibition of PAR-2 significantly enhancing 5-FU-mediated cytotoxicity and suppressing EMT signaling in CRC xenograft models (57). These preclinical findings highlight the potential of PAR-2 inhibition to augment current CRC treatment regimens and overcome therapeutic resistance.

Statins, known for their lipid-lowering effects via inhibition of HMG-CoA reductase, have emerged as promising candidates for targeting cancer pathways due to their pleiotropic actions, which extend far beyond cholesterol regulation. By disrupting the mevalonate pathway, statins interfere with protein prenylation, a process critical for the post-translational modification of key signaling molecules involved in cancer cell survival, such as Rho-GTPases (59). Statins also exhibit potent anti-inflammatory properties, which are particularly relevant in the context of CRC, where chronic inflammation is a driving force behind tumor progression. Statins suppress the production of pro-inflammatory cytokines and disrupt lipid raft formation—cholesterol-enriched microdomains within the cell membrane that are essential for cancer cell signaling (60). This disruption impairs several oncogenic pathways, including MAPK/ERK, PI3K/Akt, and NF-κB, all of which are intimately involved in CRC progression (61).

Beyond their effects on inflammation and signaling, statins have been shown to induce apoptosis and inhibit angiogenesis in various cancer models (62). Clinical evidence supports the potential of statins in reducing cancer-specific mortality and improving overall survival across several malignancies, including CRC, breast, lung, and prostate cancers (63-65). Notably, in CRC, statin use has been associated with improved outcomes, particularly

in combination with standard chemotherapeutic regimens (24,66,67). Statins have been shown to reduce all-cause mortality by 30% and cancer-specific mortality by 40%, as evidenced by observational analyses and meta-analyses (68). These effects are thought to be dose-dependent, with higher statin doses demonstrating more pronounced anti-cancer effects (69).

Rationale and knowledge gap

As discussed in the background, inflammation-driven CRC progression, mediated by PAR-2, is a critical therapeutic challenge. PAR-2 promotes tumour proliferation, invasion, and immune evasion, and its elevated expression is linked with more aggressive CRC and poor patient outcomes. While the anti-inflammatory properties of statins have been demonstrated in other contexts, no direct evidence in the literature establishes whether statins modulate PAR activity or PAR-2 signalling specifically. This gap in knowledge presents an important research opportunity.

Statins such as atorvastatin (ATV) and rosuvastatin (RSV) are known for their pleiotropic effects, including anti-inflammatory actions, but their specific roles in PAR-2 regulation within CRC have not been explored. Furthermore, the specificity of statins for PAR-2, as compared to other protease-activated receptors such as PAR-1, remains unexplored and requires further investigation. Given that previous studies have primarily focused on broad anti-inflammatory pathways like NF-κB or PI3K/Akt, a targeted understanding of statins' effect on PAR-2-mediated signalling in CRC is lacking.

Our study addresses this critical gap by evaluating whether statins selectively downregulate PAR-2 and reduce inflammation through the suppression of TNF-α secretion. The present investigation will also explore calcium signalling as a key inflammatory mediator modulated by statin treatment, providing mechanistic insights into how these agents may attenuate inflammation-driven tumour progression. Assessing the expression of PAR-1 alongside PAR-2 ensures a robust evaluation of the statins' specificity, adding depth to the findings. This targeted approach is essential for identifying statins' potential as adjunctive therapies for CRC, offering a novel therapeutic angle to mitigate cancer progression.

Objective

This study aims to evaluate the effect of ATV and RSV on PAR-2-mediated inflammatory signalling in CRC models. Specifically, we will:

(I) Investigate the dose-dependent effect of ATV and RSV on PAR-2 expression and TNF-α secretion in LPS-stimulated HT-29 and Caco-2 cells.
(II) Explore the modulation of calcium signalling by ATV and RSV as a critical mechanism for controlling inflammation.
(III) Assess the specificity of ATV and RSV for PAR-2 by comparing their effects on PAR-1 expression.

In summary, the present investigation provides new insights into the ability of statins to modulate PAR-2-related inflammatory pathways in CRC. Although this study does not assess their mechanistic role as adjunctive therapies, the results offer a foundation for future research exploring the potential clinical applications of statins in managing inflammation-driven CRC. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1027/rc).

Methods

Source of data collection and review methodology

The data for this study were derived from a dual approach integrating experimental research and a comprehensive narrative literature review. Experimental work focused on CRC cell lines, HT-29 and Caco-2, to evaluate the selective targeting of PAR-2-mediated inflammatory pathways by ATV and RSV. Inflammation was induced *in vitro* using lipopolysaccharide (LPS) as a stimulant, followed by statin treatments at varying concentrations. Molecular assays, including enzyme-linked immunosorbent assay (ELISA) for cytokine quantification, Western blotting for protein expression analysis, real-time polymerase chain reaction (PCR) for gene expression evaluation, and fluorescence imaging for calcium signalling, were employed to generate robust experimental data.

To complement and contextualize the experimental findings, a detailed narrative literature review was conducted adopting a systematic approach to identify, analyse and synthesize information investigating the role of PAR-2 in inflammation associated with cancer. The review followed a systematic methodology typically used for narrative reviews, incorporating a comprehensive and iterative search strategy. Literature searches were conducted on PubMed and other relevant databases (e.g., Scopus and Web of Science) to

identify full-text articles published in peer-reviewed journals.

Search strategy and keywords

The search was performed using predefined keywords and Boolean operators to maximize the comprehensiveness and specificity of the results. Keywords included combinations such as:

- "PAR-2" AND "inflammation" AND "cancer"
- "Protease-activated receptor 2" AND "tumorigenesis"
- "PAR-2 signalling" OR "chronic inflammation"
- "PAR-2" AND "statins" AND "anti-inflammatory effects"

Advanced search filters were applied to include only full-text articles published in English and to exclude articles with non-relevant subject areas. The search spanned the past 20 years, focusing on original research articles, reviews, and meta-analyses.

Inclusion and exclusion criteria

Articles were included if they specifically addressed the molecular mechanisms of PAR-2 in inflammation or its role in cancer progression. Studies involving clinical outcomes, cell line experiments, or relevant therapeutic interventions were prioritized. Articles lacking mechanistic insights or focused exclusively on unrelated signaling pathways were excluded.

Data extraction and synthesis

Selected articles were reviewed in detail, and relevant data were extracted on PAR-2-mediated pathways, inflammatory markers, and therapeutic modulators, including statins. The findings were synthesized to provide a cohesive narrative, highlighting both established knowledge and emerging perspectives. This literature review informed the Introduction and Discussion sections of the manuscript, ensuring a comprehensive and scientifically rigorous contextual framework for the experimental data.

By integrating experimental results with insights from a systematic narrative review, this study delivers a multi-dimensional analysis of PAR-2-driven inflammation and its implications for cancer progression and therapeutic intervention.

Cell line selection

We selected HT29 and Caco-2 cell lines to model distinct stages of CRC. HT29, derived from a grade II adenocarcinoma, represents advanced CRC with its aggressive proliferation and high EMT potential, making it ideal for studying metastasis and PAR-2-mediated signaling (70,71). Caco-2, from a well-differentiated adenocarcinoma, models early CRC and differentiates into enterocyte-like cells, allowing investigation of PAR-2's role in tumor initiation (25). Together, these lines provide a robust platform for examining therapeutic leads on PAR-2-mediated inflammation across CRC stages.

Cell culture methodology

HT29 and Caco-2 cells (Addexbio, USA) were cultured under stringent sterile conditions to maintain experimental integrity. Frozen cells were thawed in a 37 ℃ water bath and transferred to sterile 15 mL tubes containing 10 mL of pre-warmed complete medium. HT29 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine, while Caco-2 cells were grown in DMEM high glucose with 10% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acids. After centrifugation at 200 ×g for 5 minutes, the supernatants were discarded, and cell viability was assessed using Trypan Blue exclusion. Cells were seeded at 1×10^7 cells/mL in T-75 flasks and incubated at 37 ℃ with 5% CO_2. Both lines were grown to 70–80% confluence within 2–3 days, and medium was replenished every 2–3 days to ensure optimal growth. Daily microscopic observation ensured monitoring of morphology and contamination.

Subculturing protocol

At 70–80% confluence, cells were prepared for subculturing. Growth medium was aspirated, and cells were washed with sterile phosphate-buffered saline (PBS) to remove residual medium. Two millilitres of 0.25% Trypsin-EDTA (Sigma, USA) was added, followed by incubation at 37 ℃ for 2–4 minutes until detachment, confirmed via microscopy. Trypsin was neutralised with an equal volume of complete medium, and cells were centrifuged at 200 ×g for 5 minutes. The cell pellet was resuspended in fresh medium, seeded into new T-75 flasks at a 1:3 ratio, and incubated at 37 ℃ with 5% CO_2 for continued growth.

Cryopreservation of HT29 and Caco-2 cells

Cryopreservation is critical for preserving cell lines for

future use, ensuring both viability and reproducibility after thawing. Once HT29 and Caco-2 cells reached 70–80% confluence, they were trypsinized with 0.25% Trypsin-EDTA to detach them. After centrifugation at 200 ×g, cells were resuspended in cryoprotective medium containing 5% DMSO to prevent ice crystal formation during freezing. Cell density was adjusted to 1×10^6 cells/mL before aliquoting into cryovials. Gradual cooling at –1 ℃ per minute was achieved using a controlled-rate freezing container, followed by storage in liquid nitrogen for long-term preservation.

Assessment of cytotoxicity using MTT assay

We evaluated the cytotoxic effects of LPS prior to its use in inducing inflammation in HT29 and Caco-2 cells. This was done through the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Thermo Fisher Scientific, Waltham, MA, USA), following a standard protocol (72). After treating HT29 and Caco-2 cells, with different doses of LPS, and incubating with MTT solution, we measured the optical density at a wavelength of 570 nm to assess cell viability.

Inducing inflammation in HT29 and Caco-2 cells using LPS; and statin treatment

To establish an inflammatory environment in CRC models, LPS (10 µg/mL) was used to stimulate HT29 and Caco-2 cells, activating Toll-like receptor 4 (TLR4) and triggering intracellular pathways like NF-κB (72), which resulted in the secretion of pro-inflammatory cytokine (TNF-α). This mimics chronic inflammation in CRC, as has also been reported in other studies (73,74). We avoided higher LPS doses to prevent overstimulation, and excessive inflammation, which could obscure experimental outcomes (75).

After 24 hours of LPS exposure, ATV (lipophilic) was administered at 20 and 50 µg/mL concentrations while RSV (hydrophilic) was used at 10 and 20 µg/mL concentrations, to assess their differential effects on inflammatory signaling. These doses were selected based on their pharmacokinetics, with RSV being more potent at lower concentrations due to its hydrophilic nature and mode of transport that relies on transporters like organic anion-transporting polypeptides (OATPs) (76) as well as hepatic selectivity, ATV diffuses across membranes (77). Post-treatment analysis evaluated PAR-1 and PAR-2 expression, TNF-α secretion, and calcium signaling.

Assessment of TNF-α secretion using ELISA

Following the culture of HT-29 and Caco-2 cells, supernatants from treated and untreated cells were centrifuged at 3,000 rpm for 10 minutes at 4 ℃ to collect samples for ELISA. The levels of TNF-α were quantitatively assessed using a commercially available ELISA kit (Abcam, USA), following the manufacturer's protocol. Microtiter plates pre-coated with anti-TNF-α antibodies were used, and optical densities were measured at 450 nm (reference 620 nm) on a Hidex microplate reader. This allowed precise quantification of TNF-α secretion in the cell supernatants before and after treatment.

Western blot analysis for the expression of PAR-1 and PAR-2

To evaluate the expression levels of PAR-1 and PAR-2 in HT-29 and Caco-2 cells, Western blot analysis was conducted. PAR-1 was selected as a control to determine the specificity of statin-induced effects on PAR-2 for several reasons. Both PAR-1 and PAR-2 belong to the PAR family, sharing structural similarities, such as GPCR architecture and activation mechanisms via proteolytic cleavage (20). However, these receptors mediate distinct cellular responses and are activated by different proteases [PAR-1 primarily by thrombin (78) and PAR-2 by trypsin-like proteases]. These aspects have been elaborated in detail in the 'Background' of the present investigation. Despite these differences in activation, their structural homology makes PAR-1 an ideal control to assess whether statins selectively modulate PAR-2 or exert broader effects across similar GPCR family members. By evaluating PAR-1 expression, we aimed to confirm that the observed effects were specific to PAR-2, thus providing clearer insights into statin-specific targeting of inflammatory pathways.

Protein extraction and quantification

Total protein extraction was performed using a lysis buffer containing 0.5% Sodium Dodecyl Sulphate (SDS, Invitrogen, USA) supplemented with a broad spectrum of protease inhibitors to prevent protein degradation. After cell lysis, protein concentrations were quantified using the Bicinchoninic Acid (BCA) assay to ensure uniform protein loading across all experimental conditions.

Experimental design

We evaluated the effect of two statins, ATV (20 and 50 µg/mL)

and RSV (10 and 20 µg/mL), on the expression of PAR-1 and PAR-2 in inflammatory HT-29 and Caco-2 cells. Protein samples (20 µg per lane) from each condition were subjected to electrophoresis on a 10% SDS-Polyacrylamide Gel (SDS-PAGE) and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Canada) at 4 ℃.

Blocking and antibody incubation

Following the protein transfer, membranes were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline (TBS, Pierce, USA) for 60 minutes at 4 ℃. After blocking, membranes were washed with Tris-Buffered Saline containing 0.1% Tween 20 (TTBS) for 10 minutes.

For primary antibody incubation, a 1:1,000 dilution of mouse anti-human PAR-1 and PAR-2 antibodies (Santa Cruz Biotechnology, Texas, USA) was prepared in SuperBlock (Thermo Fisher Scientific), diluted 1:10 in TTBS, and incubated overnight at 4 ℃. By including PAR-1 in the analysis, we aimed to control for non-specific statin effects on other structurally similar GPCRs.

Secondary antibody and detection

After three washing cycles (10 minutes each) with TTBS, the membranes were incubated with a 1:2,000 dilution of anti-mouse IgG-HRP secondary antibody for one hour at room temperature. Protein bands were visualized using SuperSignal ULTRA Chemiluminescent Substrate (Pierce) and captured using Kodak Biomax photographic film (GE Healthcare, Canada).

Quantification

The intensity of the PAR-2 protein bands was quantified using ImageJ software to assess the relative changes in expression. By comparing PAR-1 and PAR-2 band intensities, we confirmed that statin treatment selectively affected PAR-2 without significantly altering PAR-1 levels, indicating specificity for PAR-2 in these cell lines. The results were normalized to GAPDH expression, and the ratios (Protein expression to GAPDH) were presented in one graph for comparative analysis. All Western blot experiments were run in triplicates to ensure reproducibility and reliability of the data.

RNA extraction and cDNA synthesis

Total RNA was extracted from both control and inflammatory (LPS-treated) HT-29 and Caco-2 cells before and after treatment with ATV (20 and 50 µg/mL) and RSV (10 and 20 µg/mL) using the Total RNA Isolation Kit (NZYTech, Lisbon, Portugal). RNA quality and integrity were assessed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific), ensuring consistent purity and concentration across all samples. High-quality RNA samples (with A260/A280 ratios between 1.8 and 2.0) were reverse-transcribed into complementary DNA (cDNA) using the First-Strand cDNA Synthesis Kit (OriGene, Heidelberg, Germany), according to the manufacturer's instructions (79). The resulting cDNA was used for quantitative PCR to evaluate the expression levels of target genes, including *PAR-1* and *PAR-2*, following ATV and RSV treatments.

Real-time PCR for quantification (qPCR)

qPCR assays were meticulously conducted using the QuantStudio 5 Flex Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific) to quantify the expression levels of PAR-1 and PAR-2 in HT-29 and Caco-2 cells. The reactions employed SYBR Green chemistry, which allows real-time detection of double-stranded DNA during amplification, providing a reliable and sensitive approach to measure relative gene expression.

Primers for the genes of interest (*PAR-1*, *PAR-2*, *TNF*-α and *GAPDH*) were designed using OriGene's proprietary primer design algorithm to optimize specificity and amplification efficiency. The sequences of these primers are provided in *Table 1*, demonstrating the carefully selected oligonucleotides used for targeting the genes of interest. These primers were evaluated for their specificity using *in silico* analysis, including assessments of the E-value and Bit score to ensure minimal off-target effects and high amplification precision.

All qPCR reactions were performed in technical replicates, and no-template controls were included to monitor for any potential contamination. The relative expression levels of *PAR-1* and *PAR-2* were calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as the housekeeping gene. This approach enabled the accurate quantification of gene expression changes in response to ATV and RSV treatments under inflammatory conditions, offering insights into the specific effects of statins on PAR-2 modulation and their role in CRC.

Calcium signalling assay

Calcium signalling plays a pivotal role in regulating various cellular processes, including proliferation, apoptosis,

Table 1 Oligonucleotide primers used for real time PCR for quantification

Gene	Primer type	Sequence	Accession	E-value	Bit score
GAPDH	Forward (5'-3')	GTCTCCTCTGACTTCAACAGCG	NM_002046	0.0	2,374
	Reverse (5'-3')	ACCACCCTGTTGCTGTAGCCAA			
PAR-2	Forward (5'-3')	CTCCTCTCTGTCATCTGGTTCC	NM-005242	0.0	2,861
	Reverse (5'-3')	TGCACACTGAGGCAGGTCATGA			
PAR-1	Forward (5'-3')	GCTGTCCTACTGCTTGGAAGAC	NM_022002	0.0	2,745
	Reverse (5'-3')	CTGCATCAGCACATACTCCTCC			
TNF-α	Forward (5'-3')	CTCTTCTGCCTGCTGCACTTTG	NM_000594	0.0	2,449
	Reverse (5'-3')	ATGGGCTACAGGCTTGTCACTC			

PCR, polymerase chain reaction.

and inflammatory responses, all of which are critical in cancer biology. In CRC, calcium flux is closely linked to the modulation of PAR-2 activity (80,81). ATV and RSV downregulate PAR-2 expression (see *Results* section), and investigating their impact on calcium signalling can provide valuable insights into whether their anti-inflammatory effects also influence calcium-dependent pathways. Therefore, calcium signalling assay was conducted to assess whether the modulation of PAR-2 and TNF-α by ATV and RSV, correlates with alterations in intracellular calcium levels.

Intracellular calcium signalling was assessed in HT-29 and Caco-2 cells using the calcium-sensitive dye Fluo-4, AM (Thermo Fisher Scientific). Fluo-4, AM, a cell-permeant ester, becomes highly fluorescent upon binding to intracellular calcium, providing a direct measure of calcium flux (82). Cells were cultured in 35-mm dishes containing 2 mL of complete growth medium until they reached 70–80% confluency.

Assessment was performed for control and inflammatory (LPS-treated) HT-29 and Caco-2 cells before and after treatment with ATV 20 and 50 µg/mL, and RSV 10 and 20 µg/mL. On the day of the experiment, the culture medium was removed, and cells were washed twice with pre-warmed PBS to remove serum components that could interfere with dye uptake. A 2 mL Fluo-4, AM loading solution was prepared according to the manufacturer's instructions and added to each dish. Cells were incubated for 30 minutes at 37 ℃ to allow intracellular esterases to hydrolyze the dye and promote dye uptake, followed by an additional 30-minute incubation at room temperature to complete de-esterification.

After incubation, the loading solution was discarded, and cells were washed once with PBS to remove excess dye. To maintain viability during imaging, 2 mL of fresh PBS was added to the cells. Live-cell fluorescence imaging was performed using a fluorescence microscope equipped with excitation and emission filters specific for Fluo-4 (excitation: 488 nm; emission: 516 nm). Fluorescence intensity was analysed in real-time to quantify intracellular calcium levels under the different experimental conditions.

This method provided a quantitative assessment of calcium flux across control and LPS-treated HT-29 and Caco-2 cells, both before and after treatment with ATV and RSV.

Statistical analysis

In this study, appropriate statistical methods were applied to ensure the reliability and validity of the results. All quantitative data are presented as means ± standard deviations from at least three independent experiments, each conducted in triplicate. The primary statistical approach used was the Student's *t*-test, which compared the means of two groups, typically the control and treated groups. A P value of less than 0.05 was considered statistically significant, indicating a less than 5% likelihood that the observed differences were due to chance. The *t*-test was chosen for its robustness in evaluating mean differences, particularly when working with small sample sizes, which is often the case in *in vitro* studies. Statistical analyses were carried out using GraphPad Prism software (version 9.5.1, San Diego, CA, USA), which provided accurate calculations of P values and confidence intervals. This rigorous statistical approach ensures the credibility and reproducibility of the study's conclusions.

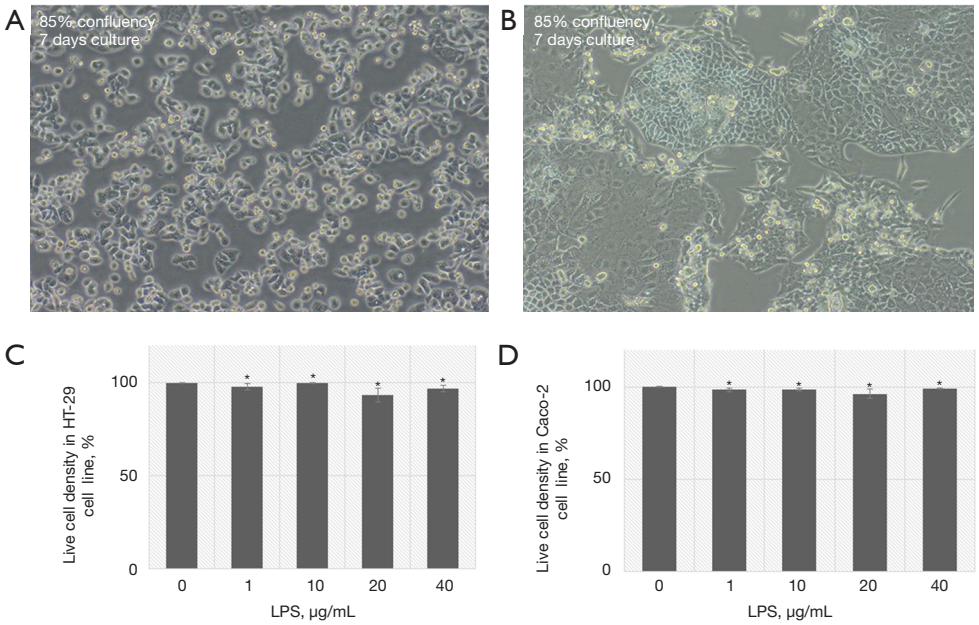

Figure 1 Morphological assessment and viability of HT-29 and Caco-2 cells after 7 days of culture. (A,B) Representative phase-contrast images of HT-29 (A) and Caco-2 (B) cells at 85% confluency after 7 days of culture in standard growth conditions. Both cell lines were observed at 20× magnification by using a phase contrast microscope. The cell lines show distinct morphological characteristics, with HT-29 cells displaying a rounded shape, while Caco-2 cells exhibit a more elongated, epithelial-like morphology with areas of tight junction formation. These images confirm healthy, confluent monolayers suitable for subsequent experimental treatments. (C,D) Quantification of cell viability assessed using the MTT assay after treatment with increasing concentrations of a test compound (1, 10, 20, and 40 µg/mL) for 24 hours. (C) HT-29 cells show a mild, non-significant reduction in viability at all concentrations, with viability remaining above 90%. (D) Caco-2 cells maintain a similar trend, with cell viability remaining high (>90%) across all treatment concentrations, indicating that the compound does not exert cytotoxic effects on these cell lines under the tested conditions. *, statistically significant differences compared to the untreated control (0 µg/mL) at $P<0.05$. These results suggest that both HT-29 and Caco-2 cells exhibit robust viability, validating their suitability for further mechanistic studies. LPS, lipopolysaccharide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide.

Results

Evaluation of LPS-induced effects on cell viability and inflammatory cancer model development in HT29 and Caco-2 cell lines

In this investigation, HT29 and Caco-2 cell lines were utilized to assess the effects of LPS on cell viability and to develop an inflammatory CRC model. *Figure 1A* represents the HT29 cells, while *Figure 1B* shows Caco-2 cells, both cultured for 7 days at 85% confluency. The images reveal healthy growth patterns in both cell lines, with HT29 cells exhibiting a well-distributed morphology, and Caco-2 cells forming their characteristic clusters (83). These observations highlight the stable and robust nature of both cell lines, demonstrating their suitability for experimentation under the conditions tested.

The MTT assay results, displayed in *Figure 1C* for HT29 and *Figure 1D* for Caco-2, indicate that there was no significant difference in cell viability at any of the tested LPS concentrations (1, 10, 20, and 40 µg/mL) when compared to the control. Both cell lines maintained strong viability across all LPS treatments, suggesting that LPS does not negatively impact their viability under these experimental conditions.

To effectively induce an inflammatory environment in the CRC models, LPS was administered at a concentration of 10 µg/mL. This treatment resulted in an increase in the secretion of the pro-inflammatory cytokine TNF-α, showing a rise of 58.47% in HT29 cells and 51.39% in Caco-2 cells (data not shown), compared to the control. These findings further validate the ability of LPS to successfully trigger an inflammatory response in both cell lines, demonstrating

its efficacy in establishing inflammation-associated CRC models.

Effect of ATV and RSV on PAR-2 expression in HT-29 cells

The effect of ATV and RSV on the expression of PAR-2 in HT-29 CRC cells was evaluated using Western blotting and reverse transcription PCR (RT-PCR) to assess both protein and mRNA levels, respectively.

Figure 2A presents the Western blot results showing PAR-2 expression in HT-29 cells under various treatment conditions. Cells were treated with LPS alone, or LPS in combination with ATV at 20 µg/mL and 50 µg/mL, and RSV at 10 and 20 µg/mL. The blots indicate a notable increase in PAR-2 expression following LPS treatment compared to untreated control cells. However, co-treatment with ATV or RSV resulted in a significant, dose-dependent decrease in PAR-2 protein expression, particularly at the higher concentrations of 50 µg/mL ATV and 20 µg/mL RSV. No changes were observed in the expression of the housekeeping gene *GAPDH*, confirming the specificity of the treatment effects on PAR-2 expression.

Figure 2B depicts the quantitative densitometric analysis of the Western blot data, normalized to GAPDH expression. Treatment with LPS alone led to a substantial increase in PAR-2 expression, with a statistically significant upregulation ($P<0.05$) compared to the control. However, co-treatment with ATV (20 and 50 µg/mL) or RSV (10 and 20 µg/mL) caused a marked reduction in PAR-2 levels in a concentration-dependent manner. The highest concentrations of both statins resulted in a significant downregulation of PAR-2 expression ($P<0.01$), further suggesting the inhibitory role of statins on PAR-2 expression in HT-29 cells.

Figure 2C shows the results of qPCR, which was employed to assess the effect of statins on PAR-2 mRNA expression in HT-29 cells. Consistent with the protein expression data, treatment with LPS significantly upregulated PAR-2 mRNA expression compared to the control ($P<0.05$). However, in the presence of ATV and RSV, there was a notable dose-dependent reduction in PAR-2 mRNA levels. Treatment with 50 µg/mL ATV or 20 µg/mL RSV significantly downregulated PAR-2 mRNA expression ($P<0.01$), further confirming the suppression of LPS-induced PAR-2 expression by these statins at the transcriptional level.

In summary, both ATV and RSV effectively attenuate LPS-induced PAR-2 expression in HT-29 cells, as demonstrated by significant reductions in PAR-2 protein and mRNA levels. These results highlight the potential of statins to modulate advantageously PAR-2-mediated inflammatory signalling pathways in CRC.

Effect of ATV and RSV on PAR-2 expression in Caco-2 cells

The effect of ATV and RSV on PAR-2 expression in Caco-2 CRC cells was evaluated using Western blotting and RT-PCR to assess both protein and mRNA levels.

Figure 3A shows the Western blot results demonstrating PAR-2 protein expression in Caco-2 cells across various treatment conditions. Cells were treated with LPS alone, or LPS in combination with ATV at 20 and 50 µg/mL, and RSV at 10 and 20 µg/mL. LPS treatment led to a significant increase in PAR-2 expression compared to control cells. However, co-treatment with ATV or RSV resulted in a concentration-dependent reduction in PAR-2 protein levels, with the highest reductions observed at 50 µg/mL ATV and 20 µg/mL RSV. GAPDH expression remained stable across all treatments, confirming the consistency of protein loading and the specificity of statin effects on PAR-2 expression.

Figure 3B presents the densitometric analysis of the Western blot data, normalized to GAPDH expression. Treatment with LPS alone caused a marked upregulation of PAR-2 expression ($P<0.05$) compared to the control group. However, co-treatment with ATV (20 and 50 µg/mL) or RSV (10 and 20 µg/mL) significantly reduced PAR-2 protein levels in a dose-dependent manner. The highest concentrations of both ATV and RSV resulted in a statistically significant reduction in PAR-2 expression ($P<0.01$), indicating the inhibitory effect of these statins on PAR-2 expression in Caco-2 cells.

Figure 3C displays the results of qPCR, assessing the impact of statins on PAR-2 mRNA expression in Caco-2 cells. In line with the protein-level data, LPS treatment alone significantly upregulated PAR-2 mRNA expression compared to the control ($P<0.05$). However, co-treatment with ATV or RSV led to a concentration-dependent reduction in PAR-2 mRNA levels. Treatment with 50 µg/mL ATV or 20 µg/mL RSV significantly downregulated PAR-2 mRNA expression ($P<0.01$), further confirming that these statins suppress LPS-induced PAR-2 expression at the transcriptional level.

In summary, both ATV and RSV effectively reduce LPS-induced PAR-2 expression in Caco-2 cells, as demonstrated by significant decreases in both protein and mRNA levels. These findings suggest that statins may modulate

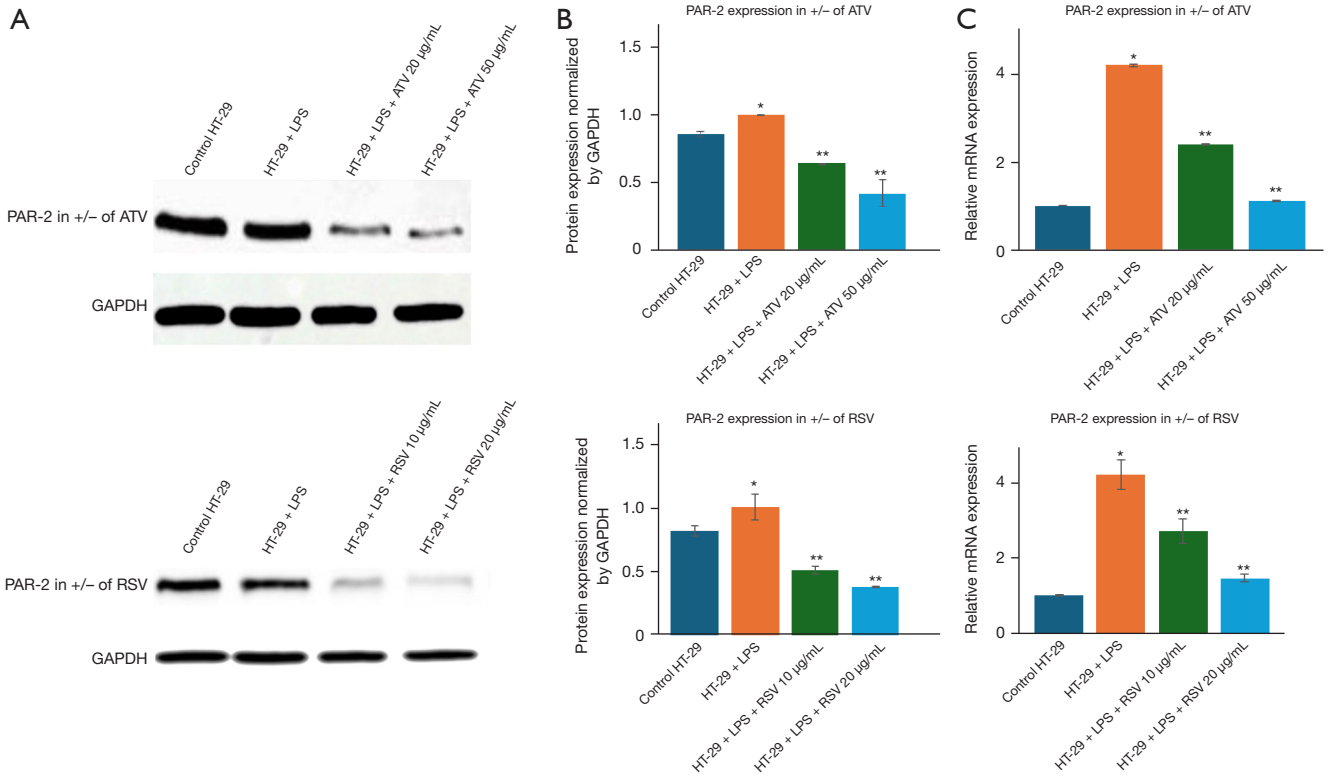

Figure 2 Modulation of *PAR-2* expression by ATV and RSV in HT-29 cells. (A) Western blot analysis showing PAR-2 protein expression in HT-29 cells under different treatment conditions. Cells were treated with LPS alone or with ATV at 20 and 50 µg/mL (top panel) and RSV at 10 and 20 µg/mL (bottom panel). LPS treatment markedly increased PAR-2 expression compared to the control. Both ATV and RSV reduced PAR-2 levels in a concentration-dependent manner, indicating statin-mediated inhibition of LPS-induced PAR-2 upregulation. GAPDH was used as a loading control to ensure consistent protein loading. (B) Densitometric quantification of PAR-2 protein levels normalized to GAPDH. Top panel (ATV): LPS significantly increased PAR-2 expression compared to the control (*, P<0.05). ATV at both 20 and 50 µg/mL significantly decreased PAR-2 expression (**, P<0.01), with the higher concentration showing a more pronounced effect. Bottom panel (RSV): LPS increased PAR-2 expression compared to the control (*, P<0.05). RSV treatment at 10 and 20 µg/mL significantly reduced PAR-2 expression (**, P<0.01), with the highest reduction observed at 20 µg/mL. (C) Relative *PAR-2* mRNA expression in HT-29 cells, measured by qPCR, presented for both ATV and RSV treatments. Top panel (ATV): LPS treatment led to a significant increase in *PAR-2* mRNA expression compared to the control (*, P<0.05). Treatment with ATV at 20 and 50 µg/mL significantly decreased *PAR-2* mRNA levels (**, P<0.01), showing a dose-dependent effect. Bottom panel (RSV): similarly, LPS increased *PAR-2* mRNA expression compared to the control (*, P<0.05). RSV at both 10 and 20 µg/mL significantly reduced *PAR-2* mRNA expression (**, P<0.01), with a stronger effect at 20 µg/mL. ATV, atorvastatin; LPS, lipopolysaccharide; qPCR, real-time polymerase chain reaction for quantification; RSV, rosuvastatin.

PAR-2-mediated inflammatory signaling pathways, offering potential therapeutic benefit in CRC.

Effect of ATV and RSV on PAR-1 expression in HT-29 cells

The effect of ATV and RSV on PAR-1 expression in HT-29 cells was evaluated using Western blotting and qPCR to assess both protein and mRNA levels. As mentioned in methodology, since PAR-1 shares structural and functional similarities with PAR-2, it was used as a control to verify the specificity of statins in modulating PAR-2 expression without affecting other PAR family receptors.

Figure 4A presents the Western blot results demonstrating PAR-1 protein expression in HT-29 cells under different treatment conditions. Cells were treated with LPS alone or co-treated with ATV at 20 and 50 µg/mL (top panel) and

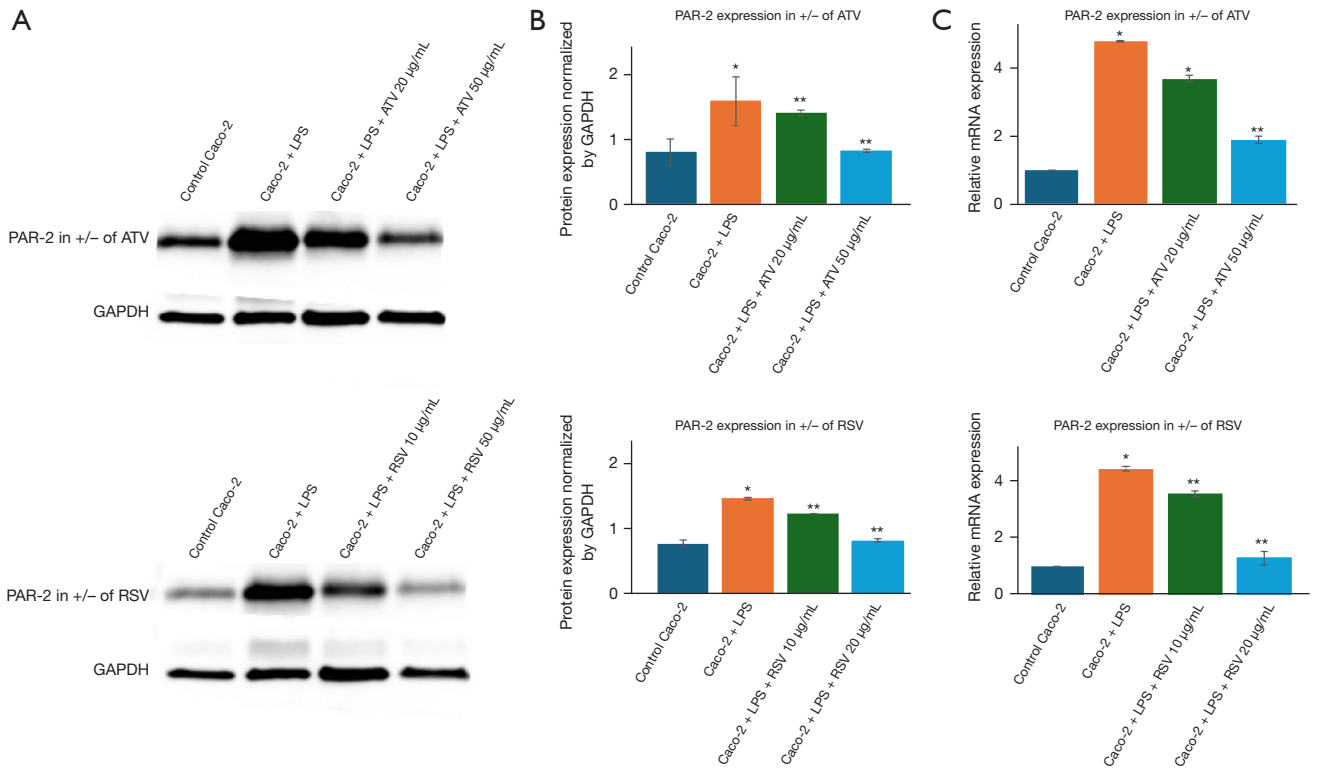

Figure 3 Modulation of *PAR-2* expression by ATV and RSV in Caco-2 cells. (A) Western blot analysis showing the expression of PAR-2 and GAPDH in Caco-2 cells under different treatment conditions. Cells were treated with LPS alone or in combination with ATV at 20 and 50 µg/mL (top panel) and RSV at 10 and 20 µg/mL (bottom panel). LPS treatment significantly increased PAR-2 protein expression compared to the control. Co-treatment with ATV and RSV resulted in a dose-dependent reduction in PAR-2 levels, with the greatest reductions observed at 50 µg/mL ATV and 20 µg/mL RSV. GAPDH was used as a loading control to ensure equal protein quantification. (B) Densitometric quantification of the Western blot bands, normalized to GAPDH expression. Top panel (ATV): LPS treatment significantly increased PAR-2 expression ($P<0.05$) compared to the control. ATV treatment at 20 and 50 µg/mL significantly reduced PAR-2 protein levels ($P<0.01$) in a concentration-dependent manner. Bottom panel (RSV): similarly, LPS treatment increased PAR-2 expression ($P<0.05$). RSV treatment at 10 and 20 µg/mL resulted in a significant reduction in PAR-2 expression ($P<0.01$), with the most pronounced effect at 20 µg/mL. (C) Relative mRNA expression of *PAR-2* in Caco-2 cells measured by qPCR. Top panel (ATV): LPS treatment significantly upregulated *PAR-2* mRNA levels ($P<0.05$) compared to the control, while ATV at both 20 and 50 µg/mL significantly reduced mRNA expression ($P<0.01$) in a dose-dependent manner. Bottom panel (RSV): LPS treatment also increased *PAR-2* mRNA levels ($P<0.05$) compared to the control. RSV at 10 and 20 µg/mL significantly downregulated *PAR-2* mRNA expression ($P<0.01$), with the greatest reduction observed at 20 µg/mL. Student's *t*-test was performed on each experimental dataset, *, $P<0.05$; **, $P<0.01$. ATV, atorvastatin; LPS, lipopolysaccharide; qPCR, real-time polymerase chain reaction for quantification; RSV, rosuvastatin.

RSV at 10 and 20 µg/mL (bottom panel). LPS treatment resulted in a slight increase in PAR-1 expression compared to the control. However, co-treatment with ATV and RSV did not significantly alter PAR-1 protein levels, indicating that the effect of statins on PAR-1 expression is negligible. GAPDH was used as a loading control to ensure equal protein quantification.

Figure 4B shows the densitometric analysis of PAR-1 protein levels, normalized to GAPDH expression. In the top panel (ATV), LPS treatment slightly upregulated PAR-1 expression compared to control cells, but co-treatment with ATV at 20 and 50 µg/mL caused only minor, non-significant changes in PAR-1 protein levels. In the bottom panel (RSV), similar to ATV, RSV treatment at 10 and 20 µg/mL resulted in minimal changes in PAR-1 expression compared to the LPS-treated group, with no statistically

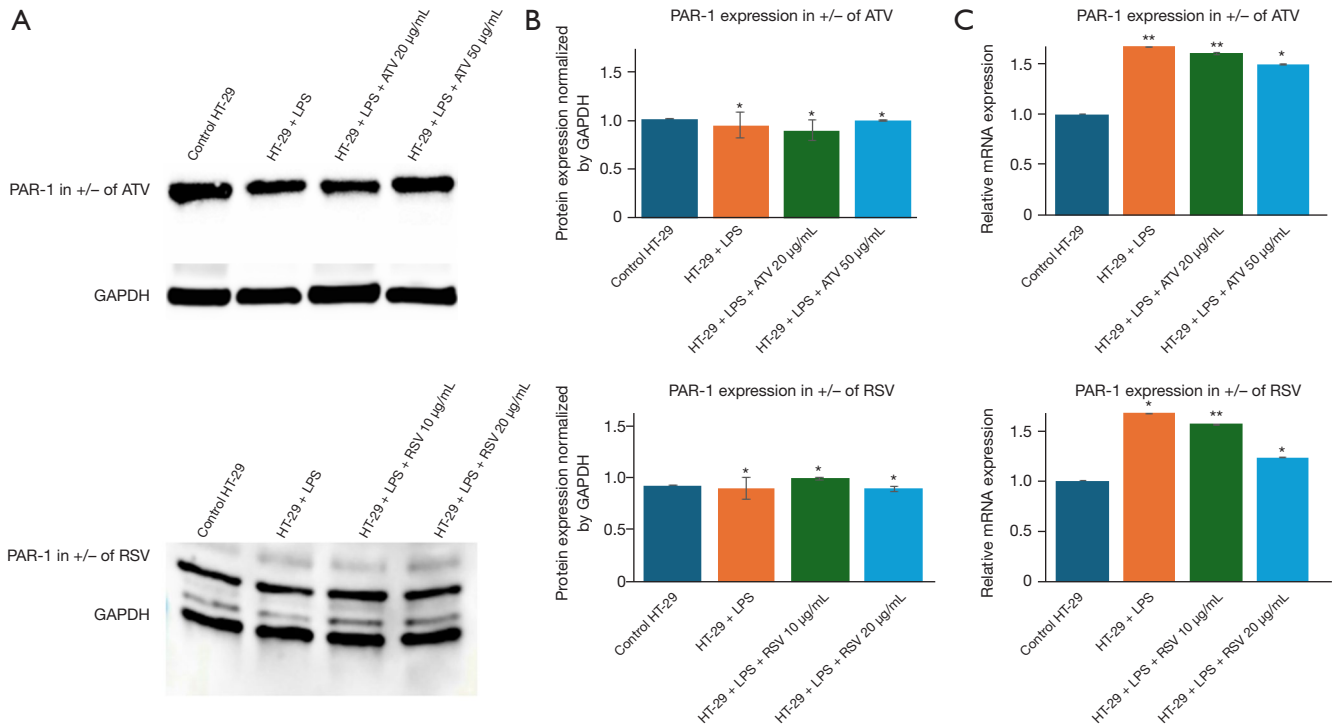

Figure 4 Modulation of *PAR-1* expression by ATV and RSV in HT-29 cells. (A) Western blot analysis showing the expression of PAR-1 and GAPDH in HT-29 cells under different treatment conditions. Cells were treated with LPS alone or in combination with ATV at 20 and 50 µg/mL (top panel) and RSV at 10 and 20 µg/mL (bottom panel). LPS treatment led to a slight increase in PAR-1 expression compared to the control. However, co-treatment with ATV or RSV resulted in only minor changes in PAR-1 levels, indicating that statins have negligible effects on PAR-1 expression. GAPDH was used as a loading control to ensure equal protein quantification. (B) Densitometric quantification of the Western blot bands, normalized to GAPDH expression. Top panel (ATV): LPS treatment slightly increased PAR-1 expression compared to the control. Treatment with ATV at 20 and 50 µg/mL resulted in minimal, non-significant changes in PAR-1 levels. Bottom panel (RSV): like ATV, RSV at 10 and 20 µg/mL showed only minor effects on PAR-1 expression, with no statistically significant differences compared to LPS treatment alone. (C) Relative mRNA expression of *PAR-1* in HT-29 cells measured by RT-PCR. Top panel (ATV): LPS treatment caused a slight increase in *PAR-1* mRNA levels compared to the control. Co-treatment with ATV at 20 and 50 µg/mL showed minor, non-significant reductions in mRNA expression. Bottom panel (RSV): LPS treatment also increased *PAR-1* mRNA levels compared to the control. RSV treatment at 10 and 20 µg/mL resulted in only slight reductions in mRNA expression, with no significant differences observed. Student's *t*-test was performed on each experimental dataset, *, P<0.05; **, P<0.01. ATV, atorvastatin; LPS, lipopolysaccharide; RSV, rosuvastatin; RT-PCR, reverse transcription time polymerase chain reaction.

significant effect.

Figure 4C presents the relative PAR-1 mRNA expression measured by RT-PCR. In the top panel (ATV), LPS treatment led to a minor increase in PAR-1 mRNA levels compared to the control. Co-treatment with ATV at both 20 and 50 µg/mL resulted in only slight, non-significant changes in mRNA levels, consistent with the protein-level findings. In the bottom Panel, RSV treatment at 10 and 20 µg/mL produced minimal effects on PAR-1 mRNA expression, further confirming that statins do not significantly regulate PAR-1 expression at the transcriptional level.

In summary, both ATV and RSV showed negligible effects on PAR-1 expression at both the protein and mRNA levels in HT-29 cells. These findings support the specificity of statins in modulating PAR-2 without influencing PAR-1 expression, confirming that the observed effects of statins are selective for PAR-2-mediated signalling pathways.

Effect of ATV and RSV on PAR-1 expression in Caco-2 cells

The impact of ATV and RSV on PAR-1 expression in Caco-2 cells was evaluated at both the protein and transcriptional levels using Western blotting and quantitative RT-PCR.

Figure 5A shows the Western blot analysis of PAR-1 expression in Caco-2 cells under different treatment conditions. Cells were treated with LPS alone or co-treated with **A**TV at 20 and 50 µg/mL (Top Panel) and RSV at 10 and 20 µg/mL (Bottom Panel). LPS treatment resulted in a slight increase in PAR-1 protein levels compared to the control. However, treatment with ATV or RSV led to only marginal changes in PAR-1 expression, indicating that statins exert negligible effects on PAR-1 protein levels. GAPDH was used as a loading control to ensure equal protein quantification.

Figure 5B presents the densitometric analysis of PAR-1 protein expression, normalized to GAPDH. In the top panel (ATV), LPS treatment caused a small increase in PAR-1 levels compared to the control. Treatment with ATV at both 20 and 50 µg/mL produced only minor changes, with no significant differences observed compared to the LPS-treated group. In the bottom panel (RSV), similar results were observed with RSV treatment, where 10 and 20 µg/mL RSV caused slight but statistically insignificant changes in PAR-1 expression compared to the LPS group.

Figure 5C shows the relative PAR-1 mRNA expression measured by qPCR in Caco-2 cells. As noted in the top panel (ATV), LPS treatment resulted in a minor increase in PAR-1 mRNA levels compared to the control. Co-treatment with ATV at 20 and 50 µg/mL produced small reductions in mRNA levels, though these changes were not statistically significant. In the bottom panel (RSV), LPS treatment also slightly increased PAR-1 mRNA levels. Treatment with RSV at 10 and 20 µg/mL produced minimal changes in mRNA expression compared to the LPS group, with no significant reductions observed.

In summary, both ATV and RSV have minimal effects on PAR-1 expression at both the protein and transcriptional levels in Caco-2 cells. These findings suggest that the statins do not significantly alter PAR-1 expression, highlighting the specificity of their effects on other signalling pathways, such as PAR-2, without impacting PAR-1-related signalling.

Effect of ATV and RSV on TNF-α secretion and mRNA levels in HT-29 Cells

The effect of ATV and RSV on TNF-α secretion and transcription was evaluated using ELISA and RT-PCR, respectively.

Figure 6A,*6B* present the ELISA results showing the dose-dependent effect of ATV and RSV on TNF-α secretion in LPS-stimulated HT-29 cells. In *Figure 6A*, LPS treatment resulted in a marked increase in TNF-α secretion compared to the control. Co-treatment with ATV at 10, 20, 50, and 100 µg/mL caused a dose-dependent reduction in TNF-α secretion, with significant reductions observed at concentrations of 20 µg/mL and above ($P<0.01$). In *Figure 6B*, LPS treatment also increased TNF-α secretion. RSV treatment at 5, 10, 20, and 50 µg/mL produced a dose-dependent reduction in TNF-α secretion, with significant suppression achieved from 10 µg/mL onwards ($P<0.01$).

Figure 6C,*6D* present the RT-PCR results, reflecting the effect of ATV and RSV on TNF-α mRNA expression. In *Figure 6C*, LPS stimulation significantly increased TNF-α mRNA expression compared to the control ($P<0.05$). Co-treatment with ATV at 20 and 50 µg/mL significantly reduced TNF-α mRNA levels in a dose-dependent manner ($P<0.01$). In *Figure 6D*, LPS treatment also elevated TNF-α mRNA expression compared to the control ($P<0.05$). Treatment with RSV at 10 and 20 µg/mL resulted in significant reductions in TNF-α mRNA levels ($P<0.01$), consistent with the dose-dependent pattern observed in the ELISA results.

In summary, both ATV and RSV significantly suppress LPS-induced TNF-α secretion and mRNA expression in HT-29 cells. These findings demonstrate the anti-inflammatory potential of statins by inhibiting TNF-α-mediated signalling, with higher concentrations of ATV and RSV producing more pronounced effects.

Effect of ATV and RSV on TNF-α secretion and mRNA levels in Caco-2 cells

The impact of ATV and RSV on TNF-α secretion and transcription was evaluated using ELISA and RT-PCR in LPS-stimulated Caco-2 cells.

Figure 7A shows the ELISA results for TNF-α secretion in Caco-2 cells treated with LPS alone or with increasing concentrations of ATV at 10, 20, 50, and 100 µg/mL. LPS treatment resulted in a substantial increase in TNF-α secretion compared to the control. Co-treatment with ATV caused a dose-dependent reduction in TNF-α secretion, with significant reductions observed at 20 µg/mL and higher concentrations ($P<0.01$). The highest suppression was achieved at 50 and 100 µg/mL of ATV.

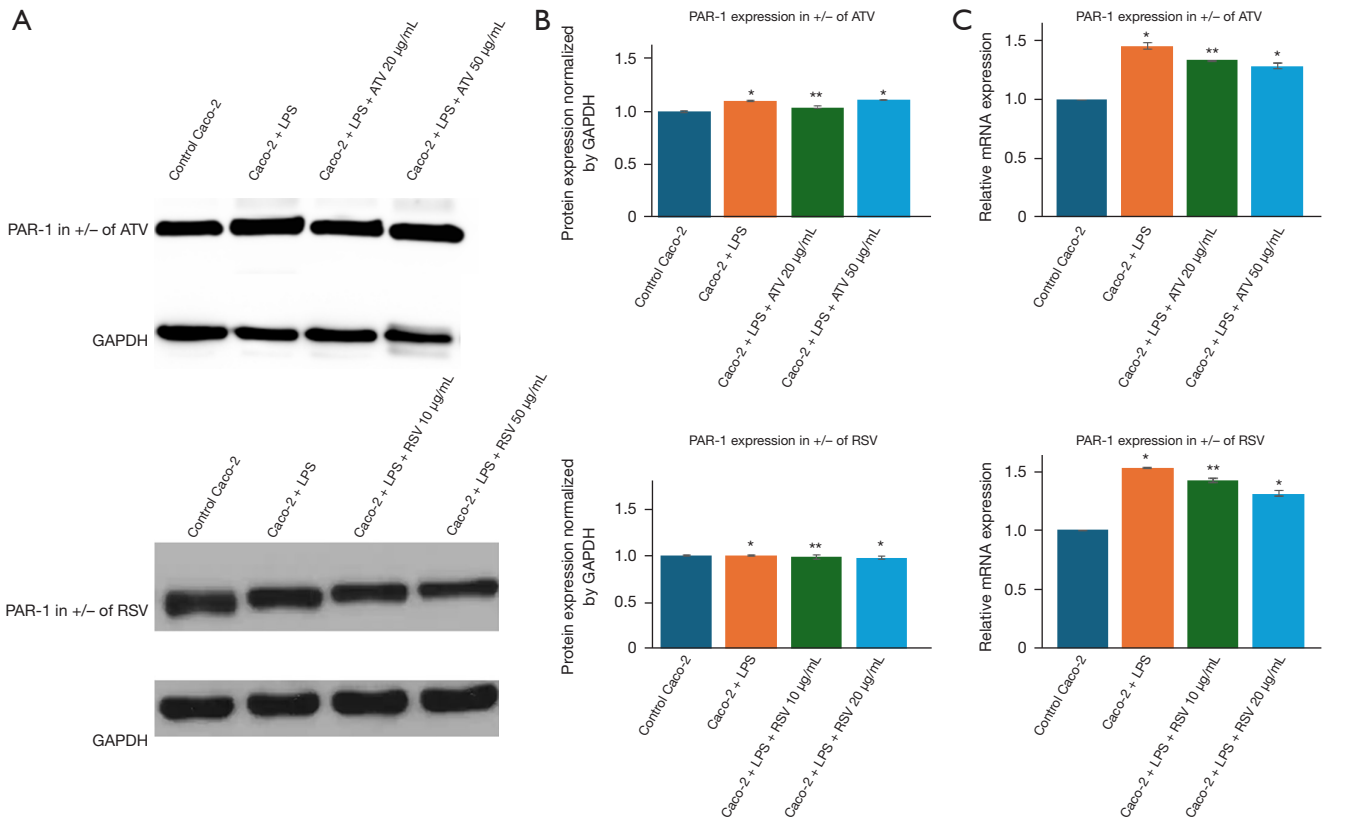

Figure 5 Modulation of *PAR-1* expression by ATV and RSV in Caco-2 cells. (A) Western blot analysis showing PAR-1 protein expression in Caco-2 cells under different treatment conditions. Cells were treated with LPS alone or in combination with ATV at 20 and 50 µg/mL (top panel) and RSV at 10 and 20 µg/mL (bottom panel). LPS treatment slightly increased PAR-1 expression compared to the control. However, co-treatment with ATV or RSV produced only minor changes in PAR-1 levels, indicating that statins have minimal effects on PAR-1 expression. GAPDH was used as a loading control to ensure consistent protein loading and quantification. (B) Densitometric quantification of the Western blot bands, normalized to GAPDH expression. Top panel (ATV): LPS treatment caused a slight increase in PAR-1 expression ($P<0.05$) compared to the control. Treatment with ATV at 20 and 50 µg/mL resulted in minor, non-significant changes in PAR-1 levels. Bottom panel (RSV): Like ATV, RSV treatment at 10 and 20 µg/mL showed minimal effects on PAR-1 expression, with no significant changes compared to the LPS-treated group. (C) Relative *PAR-1* mRNA expression in Caco-2 cells measured by qPCR. Top panel (ATV): LPS treatment led to a small increase in *PAR-1* mRNA expression ($P<0.05$) compared to the control. Treatment with ATV at both concentrations caused only minor reductions in mRNA levels, which were not statistically significant. Bottom panel (RSV): LPS also increased *PAR-1* mRNA expression slightly compared to the control. RSV treatment at 10 and 20 µg/mL resulted in minimal changes in mRNA expression, with no statistically significant differences compared to the LPS group. Student's *t*-test was performed on each experimental dataset, *, $P<0.05$; **, $P<0.01$. ATV, atorvastatin; LPS, lipopolysaccharide; RSV, rosuvastatin; qPCR, real-time polymerase chain reaction for quantification.

Figure 7B presents the ELISA results for TNF-α secretion following treatment with RSV at 5, 10, 20, and 50 µg/mL. LPS stimulation significantly increased TNF-α secretion compared to the control. RSV treatment produced a dose-dependent decrease in TNF-α secretion, with significant reductions starting from 10 µg/mL ($P<0.01$). The greatest reduction was observed at 50 µg/mL RSV.

Figure 7C,7D depict the RT-PCR results reflecting the effect of ATV and RSV on TNF-α mRNA expression. LPS treatment significantly upregulated TNF-α mRNA levels compared to the control ($P<0.05$). In cells co-treated with ATV, a dose-dependent reduction in TNF-α mRNA expression was observed, with significant suppression at 20 and 50 µg/mL ATV ($P<0.01$). Similarly, RSV treatment

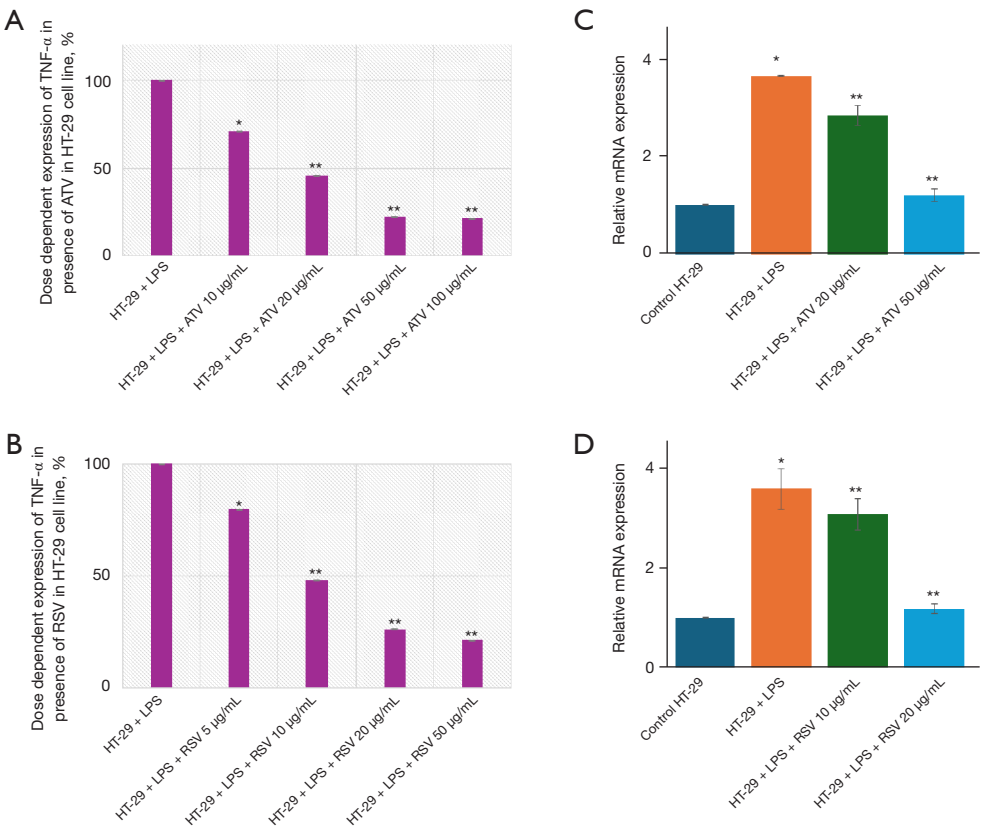

Figure 6 Dose-dependent modulation of *TNF*-α secretion and mRNA expression by ATV and RSV in HT-29 cells. (A) TNF-α secretion measured by ELISA in HT-29 cells treated with LPS alone or in combination with ATV at concentrations of 10, 20, 50, and 100 µg/mL. LPS treatment alone resulted in a significant increase in TNF-α secretion. Co-treatment with ATV produced a dose-dependent reduction in TNF-α secretion, with significant suppression observed at 20 µg/mL and higher concentrations (P<0.01). (B) TNF-α secretion measured by ELISA following treatment with LPS and RSV at 5, 10, 20, and 50 µg/mL. RSV treatment resulted in a dose-dependent reduction in TNF-α secretion, with significant decreases starting from 10 µg/mL (P<0.01) and maximum suppression observed at 50 µg/mL RSV. (C) Relative mRNA expression of *TNF*-α measured by RT-PCR in HT-29 cells treated with LPS and ATV. LPS treatment significantly increased *TNF*-α mRNA expression compared to the control (P<0.05). Co-treatment with ATV at 20 and 50 µg/mL resulted in significant reductions in *TNF*-α mRNA levels (P<0.01), confirming the dose-dependent inhibitory effect of ATV. (D) *TNF*-α mRNA expression in HT-29 cells treated with LPS and RSV, measured by RT-PCR. LPS treatment elevated *TNF-α* mRNA expression (P<0.05). RSV treatment at 10 and 20 µg/mL significantly reduced TNF-α mRNA expression (P<0.01), demonstrating the dose-dependent effect of RSV on *TNF-α* transcription. Student's *t*-test was performed on each experimental dataset, *, P<0.05; **, P<0.01. ATV, atorvastatin; ELISA, enzyme linked immunosorbent assay; LPS, lipopolysaccharide; RSV, rosuvastatin; RT-PCR, reverse transcription time polymerase chain reaction.

significantly reduced TNF-α mRNA expression in a concentration-dependent manner, with the most significant reduction observed at 20 µg/mL (P<0.01).

In summary, both ATV and RSV effectively inhibit LPS-induced TNF-α secretion and mRNA expression in Caco-2 cells. The results suggest that ATV and RSV modulate TNF-α-mediated inflammation, providing therapeutic relevance in the context of CRC.

Effect of ATV and RSV on calcium signalling in HT-29 cells

The impact of ATV and RSV on calcium signalling in HT-29 cells was assessed by measuring fluorescence intensity using the Fluo-4 AM dye. *Figure 8A-8F* presents the fluorescence images of HT-29 cells under different treatment conditions, with *Figure 8G* providing the corresponding quantification of fluorescence intensity.

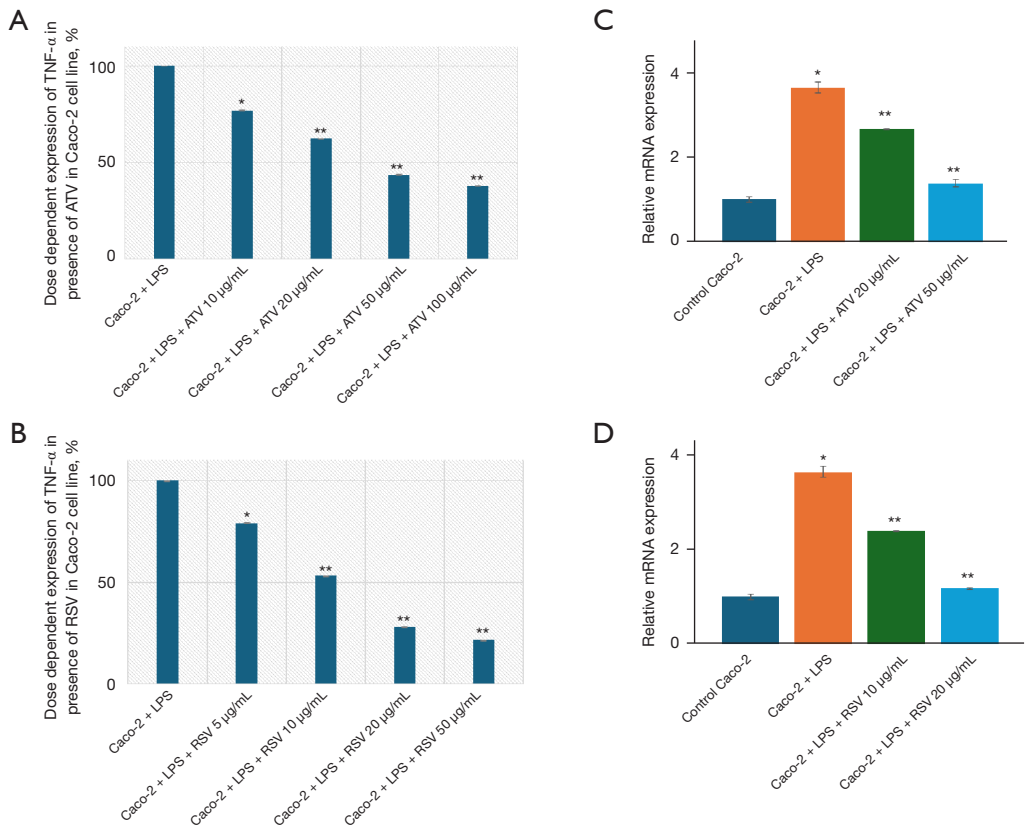

Figure 7 Dose-dependent modulation of TNF-α secretion and mRNA expression by ATV and RSV in Caco-2 cells. (A) TNF-α secretion measured by ELISA in Caco-2 cells treated with LPS alone or co-treated with ATV at concentrations of 10, 20, 50, and 100 µg/mL. LPS treatment resulted in a significant increase in TNF-α secretion compared to the control. Co-treatment with ATV caused a dose-dependent reduction in TNF-α secretion, with significant reductions observed at 20 µg/mL and above (P<0.01). The highest suppression was observed at 100 µg/mL ATV. (B) TNF-α secretion measured by ELISA in Caco-2 cells following treatment with LPS and RSV at 5, 10, 20, and 50 µg/mL. LPS stimulation led to a marked increase in TNF-α secretion. RSV treatment produced a dose-dependent reduction in TNF-α secretion, with statistically significant reductions starting from 10 µg/mL (P<0.01). The greatest suppression was observed at 50 µg/mL RSV. (C) Relative mRNA expression of *TNF-α* measured by RT-PCR in Caco-2 cells treated with LPS and ATV. LPS treatment significantly increased *TNF-α* mRNA levels compared to the control (P<0.05). Co-treatment with ATV at 20 and 50 µg/mL led to a dose-dependent reduction in *TNF-α* mRNA expression, with significant suppression observed at both concentrations (P<0.01). (D) *TNF-α* mRNA expression measured by RT-PCR in Caco-2 cells treated with LPS and RSV. LPS treatment significantly elevated *TNF-α* mRNA levels compared to the control (P<0.05). RSV treatment at 10 and 20 µg/mL produced a dose-dependent decrease in *TNF-α* mRNA expression, with the highest reduction observed at 20 µg/mL (P<0.01). Student's *t*-test was performed on each experimental dataset, *, P<0.05; **, P<0.01. ATV, atorvastatin; ELISA, enzyme linked immunosorbent assay; LPS, lipopolysaccharide; RSV, rosuvastatin; RT-PCR, reverse transcription time polymerase chain reaction.

Figure 8A shows the baseline fluorescence in control HT-29 cells, displaying minimal fluorescence intensity. *Figure 8B* illustrates the effect of LPS treatment alone, which led to a marked increase in calcium fluorescence intensity, indicating enhanced intracellular calcium levels in response to inflammation.

Figure 8C,8D present the fluorescence intensities in HT-29 cells treated with LPS in combination with 20 µg/mL and 50 µg/mL of ATV, respectively. A dose-dependent reduction in fluorescence intensity was observed with increasing concentrations of ATV, suggesting that ATV mitigates calcium influx induced by LPS stimulation.

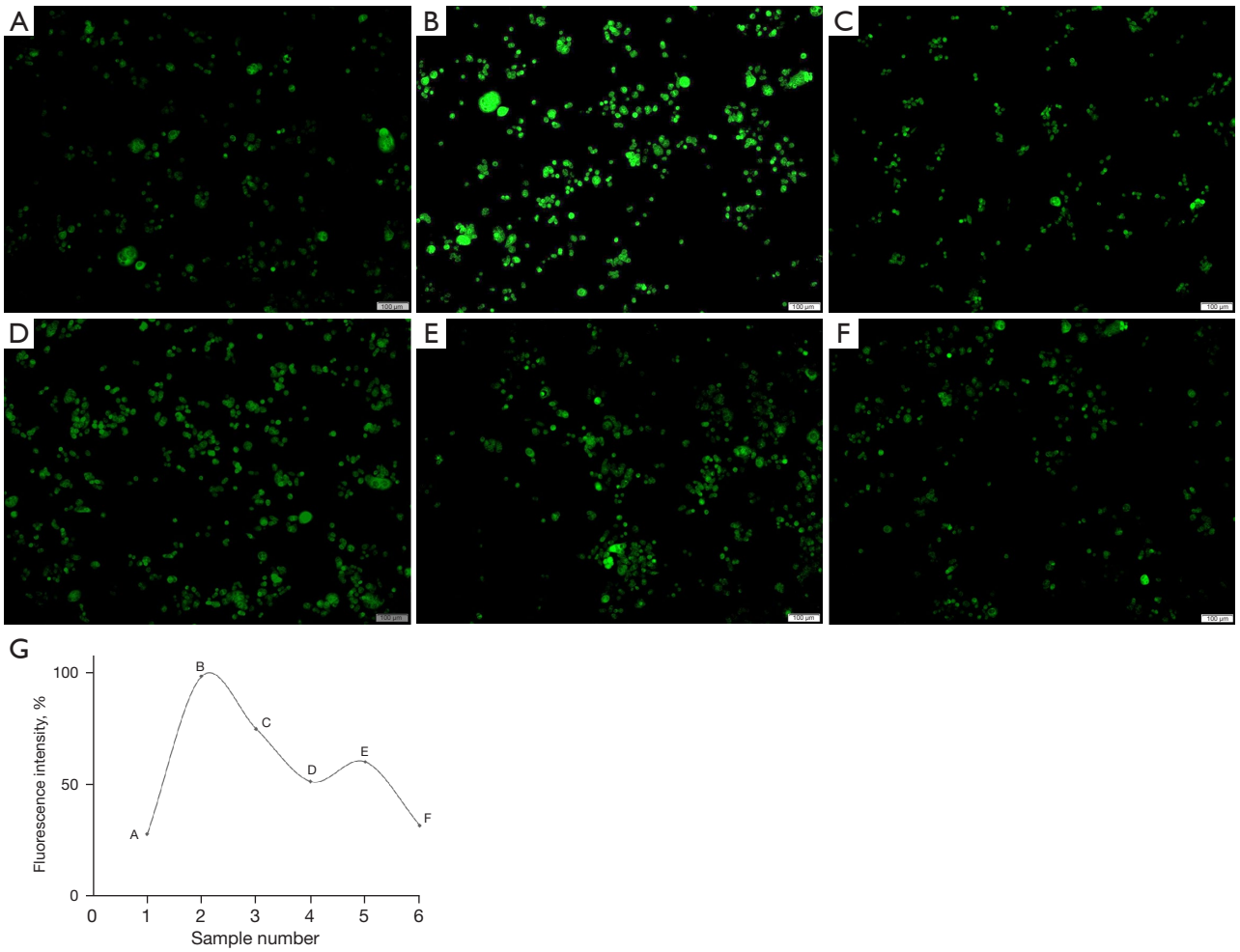

Figure 8 Effect of ATV and RSV on calcium fluorescence intensity in HT-29 cells. (A-F) Fluorescence microscopy images at 20× magnification of HT-29 cells stained with Fluo-4 AM, depicting calcium fluorescence under different treatment conditions. (A) Control HT-29 cells showing minimal baseline fluorescence. (B) HT-29 cells treated with LPS, displaying enhanced fluorescence intensity, indicative of increased intracellular calcium levels. (C) Cells treated with LPS + ATV (20 µg/mL), showing a reduction in fluorescence compared to LPS treatment alone. (D) Cells treated with LPS + ATV (50 µg/mL), exhibiting further suppression of fluorescence, indicating a dose-dependent reduction in calcium influx. (E) HT-29 cells treated with LPS + RSV (10 µg/mL), with fluorescence intensity slightly reduced compared to LPS alone. (F) Cells treated with LPS + RSV (20 µg/mL), displaying the lowest fluorescence among RSV-treated groups, demonstrating a dose-dependent inhibitory effect on calcium signalling. (G) Quantified fluorescence intensity of HT-29 cells across treatment conditions (A-F); the highest fluorescence is observed with LPS treatment alone, reflecting elevated calcium levels; co-treatment with ATV or RSV reduces fluorescence in a dose-dependent manner, with the most substantial suppression seen at 50 µg/mL ATV and 20 µg/mL RSV. ATV, atorvastatin; LPS, lipopolysaccharide; RSV, rosuvastatin.

Figure 8E,8F display the fluorescence intensities in cells treated with LPS along with 10 µg/mL and 20 µg/mL of RSV, respectively. RSV also demonstrated a dose-dependent reduction in calcium fluorescence, with lower fluorescence levels at 20 µg/mL RSV compared to 10 µg/mL, indicating the inhibitory effect of RSV on calcium signalling.

Figure 8G provides the quantified fluorescence intensity from Panels A to F, confirming the dose-dependent reduction in calcium fluorescence with both ATV and RSV treatments. LPS alone elicited the highest fluorescence intensity, while the addition of ATV and RSV resulted in significant reductions, consistent with their roles in

modulating calcium-dependent signalling pathways.

In summary, both ATV and RSV effectively reduce LPS-induced calcium signalling in HT-29 cells, as evidenced by the dose-dependent decreases in fluorescence intensity. These findings highlight the potential of statins to inhibit calcium-dependent inflammatory responses in CRC.

Effect of ATV and RSV on calcium signalling in Caco-2 cells

The impact of ATV and RSV on calcium signalling in Caco-2 cells was assessed by measuring fluorescence intensity using the Fluo-4 AM dye. *Figure 9A-9F* presents fluorescence microscopy images of Caco-2 cells under various treatment conditions, with the corresponding quantification of fluorescence intensity presented in *Figure 9G*.

Figure 9A shows minimal baseline fluorescence in control Caco-2 cells, indicating low intracellular calcium levels. *Figure 9B* illustrates a significant increase in fluorescence intensity following LPS treatment alone, suggesting heightened intracellular calcium concentration due to inflammatory stimulation.

Figure 9C,9D depict the effects of ATV at concentrations of 20 µg/mL and 50 µg/mL, respectively, in LPS-stimulated cells. Both treatments show a decrease in fluorescence intensity compared to LPS alone, with 50 µg/mL ATV exhibiting a more pronounced reduction, indicating a dose-dependent inhibition of calcium signalling.

Figure 9E,9F display the results of RSV treatment at 10 and 20 µg/mL, respectively. RSV also induced a dose-dependent reduction in fluorescence intensity, with 20 µg/mL RSV demonstrating the most substantial suppression of calcium signalling compared to LPS treatment alone.

Figure 9G provides the quantified fluorescence intensity across the treatment conditions from Panels A to F. LPS treatment led to the highest fluorescence intensity, consistent with elevated intracellular calcium. Co-treatment with ATV or RSV resulted in progressive, dose-dependent reductions in fluorescence, with the greatest inhibitory effect observed at the highest concentrations of each statin.

In summary, ATV and RSV effectively attenuate LPS-induced calcium signalling in Caco-2 cells, as evidenced by dose-dependent reductions in fluorescence intensity. These findings highlight the ability of both statins to modulate calcium-dependent inflammatory pathways in CRC models.

Discussion

Key findings

This study demonstrates that ATV and RSV selectively inhibit PAR-2 expression and TNF-α secretion in LPS-stimulated CRC cell lines, HT-29 and Caco-2, providing novel insights into the anti-inflammatory actions of statins. Our results show that both statins produce a dose-dependent reduction in PAR-2 expression at both the mRNA and protein levels, with the highest inhibition observed at 50 µg/mL ATV and 20 µg/mL RSV (*Figures 2,3*). The suppression of TNF-α secretion (*Figures 6,7*), further corroborates the inhibitory effect of these statins on inflammation-mediated signalling pathways.

Importantly, the specificity of ATV and RSV towards PAR-2 was confirmed by the negligible impact on PAR-1 expression in both CRC cell lines (*Figures 4,5*). This specificity distinguishes the effects of ATV and RSV, demonstrating that the statins modulate PAR-2-driven inflammatory pathways without altering other related protease-activated receptor signalling, such as PAR-1. This novel aspect of selective targeting provides crucial mechanistic insight and highlights the potential for minimizing off-target effects.

Furthermore, our calcium fluorescence experiments using Fluo-4 AM dye reveal that ATV and RSV inflammation-induced calcium influx in a concentration-dependent manner (*Figure 8*). This modulation of calcium signalling, a key player in inflammation and tumour progression, adds a mechanistic dimension to the anti-inflammatory effects of these statins. Collectively, these findings suggest that ATV and RSV mitigate calcium-dependent signalling pathways, contributing to the reduction of inflammation in CRC models.

Although this study does not explore the role of statins as adjunctive therapies in clinical settings, it provides foundational evidence supporting their potential therapeutic relevance in CRC. The selective targeting of PAR-2 and the suppression of TNF-α secretion, along with reduced calcium signalling, underscore the unique anti-inflammatory properties of statins in CRC. These results offer promising avenues for further research aimed at evaluating the translational potential of statins in managing inflammation-driven CRC progression.

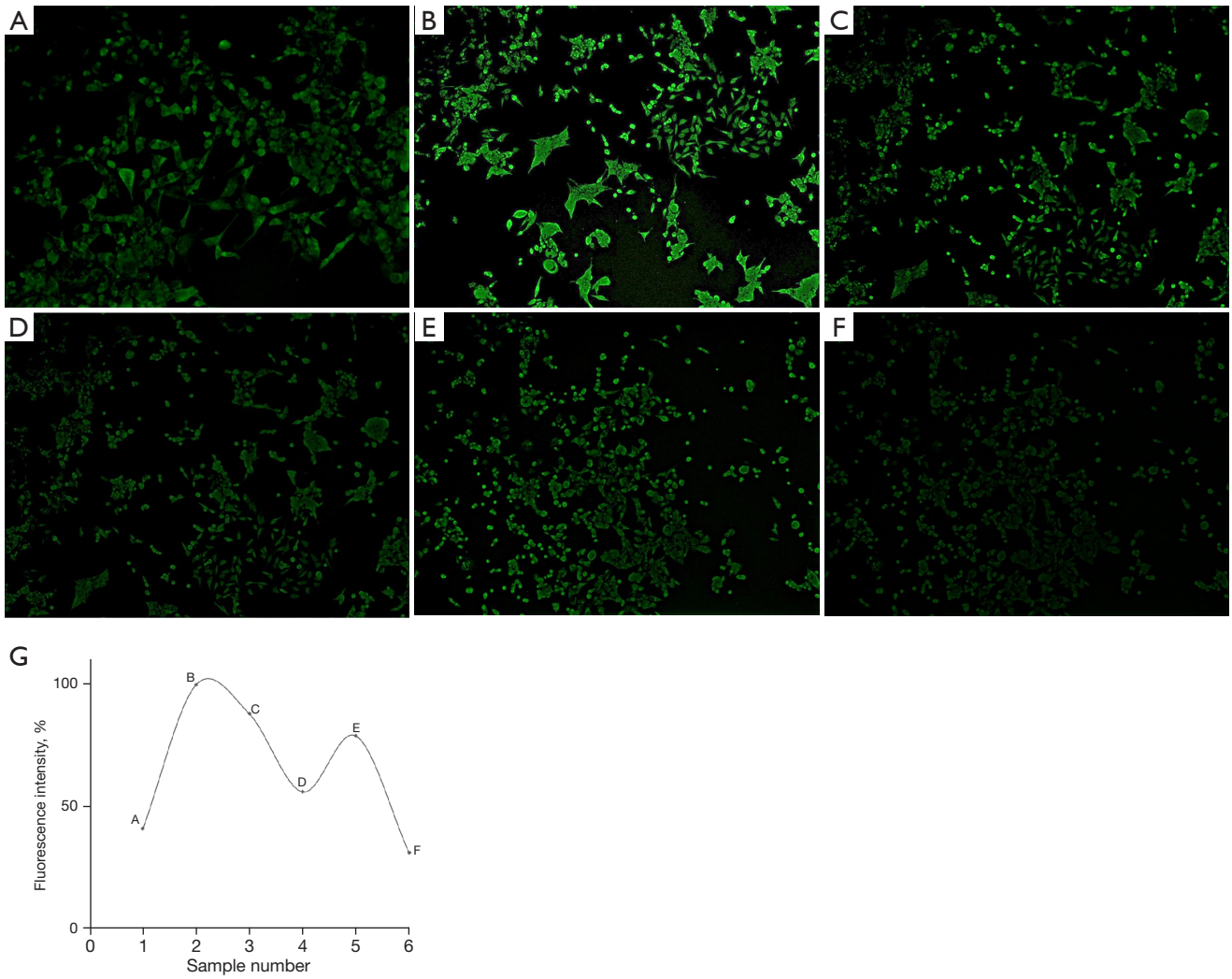

Figure 9 Effect of ATV and RSV on calcium fluorescence intensity in Caco-2 cells. (A-F) Fluorescence microscopy images of Caco-2 cells at 20× magnification stained with Fluo-4 AM, representing calcium fluorescence under different treatment conditions. (A) Control Caco-2 cells showing low baseline fluorescence. (B) Cells treated with LPS, exhibiting a substantial increase in fluorescence intensity, indicating elevated intracellular calcium levels. (C) Cells treated with LPS + ATV (20 µg/mL), showing a reduction in fluorescence compared to LPS alone. (D) Cells treated with LPS + ATV (50 µg/mL), with further suppression of fluorescence, demonstrating a dose-dependent effect. (E) Cells treated with LPS + RSV (10 µg/mL), showing moderate reduction in fluorescence. (F) Cells treated with LPS + RSV (20 µg/mL), displaying the lowest fluorescence intensity among RSV-treated groups, indicating the most significant reduction in calcium influx. (G) Quantification of fluorescence intensity across the treatment groups from Panels A to F; the highest fluorescence intensity was observed in LPS-treated cells, indicating elevated calcium signalling; co-treatment with ATV or RSV resulted in a dose-dependent decrease in fluorescence intensity, with the most notable reductions at 50 µg/mL ATV and 20 µg/mL RSV. ATV, atorvastatin; LPS, lipopolysaccharide; RSV, rosuvastatin.

Strengths and limitations

Strengths

Statin specificity

This study provides valuable insights into the differential effects of lipophilic (ATV) and hydrophilic (RSV) statins on PAR-2 signalling in CRC cell lines. The physicochemical properties of these statins play a critical role in determining their intracellular distribution and therapeutic action. Lipophilic statins such as ATV (84), can diffuse across lipid membranes, enhancing their ability to interact with

intracellular targets, including endoplasmic reticulum (ER) stress pathways and NF-κB signalling, both of which are implicated in CRC progression. Lipophilic statins may also disrupt the lipid rafts that harbour PAR-2 (85), reducing receptor availability on the cell surface and blunting inflammatory signalling. Conversely, hydrophilic statins like RSV, which are selectively taken up by hepatocytes through OATPs (86), have limited systemic distribution. This hepato-selectivity reduces the risk of off-target effects and systemic toxicity. The distinct pharmacological profiles of these statins highlight the potential for ATV to be more effective in extrahepatic tumours like CRC, while RSV could offer safer therapeutic options with fewer side effects. The comparative analysis informs future personalized therapies, suggesting that lipophilic statins may be preferred for targeting non-hepatic malignancies like CRC.

Cell line diversity

The use of HT-29 and Caco-2 cell lines enhances the robustness of this study. HT-29 cells represent an undifferentiated and aggressive CRC phenotype, known to exhibit high proliferation rates and chemoresistance, mimicking advanced-stage tumours (87,88). In contrast, Caco-2 cells can differentiate into enterocyte-like cells, more closely resembling normal colonic epithelium (89). This diversity enables a broader understanding of statin effects across different stages of tumour development and differentiation states. HT-29 cells, for example, provide insight into how statins might affect highly proliferative, resistant tumours, while Caco-2 cells model the effects of statins on less aggressive or early-stage tumours. This dual-model approach ensures that the findings are not restricted to a single cell phenotype and can inform stage-specific therapeutic interventions in CRC.

PAR-2 specificity

This study's focus on PAR-2 downregulation without affecting PAR-1 represents a significant advancement. PAR-2, often overexpressed in CRC, drives inflammation by activating pathways such as MAPK/ERK1/2 and PI3K/Akt, promoting tumour growth, invasion, and metastasis. Statin-mediated inhibition of PAR-2 could interrupt these pro-tumorigenic pathways, thereby attenuating inflammation-driven CRC progression. Importantly, PAR-1, which is predominantly involved in normal coagulation and vascular integrity (90), remains unaffected, minimizing the risk of disrupting normal haemostatic processes. This receptor specificity offers a safer therapeutic profile, as it avoids unintended side effects, such as increased bleeding risk, which might arise from PAR-1 inhibition. This precision targeting of PAR-2 distinguishes statins as potential adjuncts to CRC therapy, providing a mechanistically targeted approach to reduce tumour-promoting inflammation.

Comprehensive molecular analysis

The study employs a multi-level molecular approach, combining transcriptional (RT-PCR), protein expression (Western blot), and functional (calcium fluorescence imaging) analyses. This integrative methodology allows for a thorough examination of statin effects on PAR-2 signalling at multiple biological levels. The inclusion of calcium fluorescence assays further strengthens the mechanistic understanding by linking statins to the suppression of calcium-dependent signalling pathways, which play essential roles in cell proliferation, migration, and inflammation. By demonstrating a dose-dependent reduction in calcium influx, the study suggests that statins inhibit calcium signalling cascades linked to PAR-2 activation, providing further insight into their therapeutic potential in CRC.

Limitations

In vitro models and tumour microenvironment (TME)

Although HT-29 and Caco-2 cells are well-established models, they lack the complexity of *in vivo* systems, including interactions with immune cells, fibroblasts, and extracellular matrix components present in the TME (91). These interactions are critical for understanding tumour progression, angiogenesis, and immune evasion. For example, tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), which are absent in these models, play significant roles in CRC progression (92). Future studies should incorporate co-culture systems (93), organoids (94), or xenograft models (95) to better capture the interplay between CRC cells and the TME.

Genetic and epigenetic variability

The study relies on two CRC cell lines, HT-29 and Caco-2, which have specific genetic and epigenetic profiles that may limit the generalizability of the findings. HT-29 cells harbour mutations in APC and TP53, which are common in CRC (96), while Caco-2 cells exhibit relatively stable genomes but differ in their differentiation capacity (97). However, CRC tumours exhibit substantial genetic and epigenetic heterogeneity across patients, including mutations in genes such as *KRAS*, *BRAF*, and *PI3KCA* (98). Epigenetic factors, including methylation of tumour suppressor genes and histone modifications, also influence tumour behaviour and treatment response. These variabilities could impact how CRC tumours respond to statin treatment, suggesting that future studies

should explore the role of tumour genotype and epigenetic landscape in determining statin sensitivity.

Pharmacokinetics and tissue distribution

This study does not address the pharmacokinetic behaviour of statins in CRC, including how first-pass metabolism, plasma protein binding, and tissue-specific distribution influence their bioavailability at the tumour site. Lipophilic statins like ATV may accumulate in lipid-rich tissues, potentially reducing their availability in tumours, while hydrophilic statins like RSV exhibit restricted tissue penetration, which could limit their therapeutic efficacy. Further pharmacokinetic studies, including *in vivo* models, are needed to better understand how statins distribute within the body and accumulate in tumours.

Long-term effects and resistance mechanisms

The study focuses on acute responses to statin treatment, limiting insights into potential long-term effects. Chronic statin exposure might trigger compensatory feedback loops or lead to the development of treatment resistance through upregulation of survival pathways, such as PI3K/Akt or ERK1/2 signalling (99,100). Understanding these compensatory mechanisms will be essential for developing durable therapeutic strategies. Future studies should assess long-term statin exposure in CRC models to determine whether resistance emerges and how it can be mitigated.

Mechanistic depth of PAR-2 modulation

While this study effectively demonstrates PAR-2 downregulation through statin treatment, further exploration of the impacted downstream signalling pathways is essential for a more comprehensive understanding. PAR-2 is known to regulate several signalling cascades critical for CRC progression and inflammation, including the ERK1/2, NF-κB, GRKs, PI3K/Akt, and β-catenin pathways (*Figure 10*), all discussed in the introduction.

The ERK1/2 pathway, activated through both G-protein-dependent and β-arrestin mechanisms, promotes cellular proliferation, migration, and survival, contributing to tumour progression (*Figure 10*). Statins potentially interfere with this pathway, reducing cancer-promoting signals. Similarly, the NF-κB pathway, responsible for producing pro-inflammatory cytokines like TNF-α, is a key target. Attenuating NF-κB through PAR-2 modulation by statins aligns with the reductions in TNF-α secretion observed in this study (*Figures 6*,*7*).

In addition, PI3K/Akt signalling, which supports survival and proliferation under inflammatory conditions, may be downregulated by statins, mitigating tumor growth and chemoresistance. The introduction also discusses β-catenin-mediated transcription, regulated through PAR-2 interactions, which promotes CSC renewal and EMT. Statins' ability to reduce PAR-2 activity could diminish β-catenin stability, thereby limiting metastatic potential and tumour aggressiveness.

While this study focuses on PAR-2 and minimal PAR-1 impact, future research should explore the possible roles of other PAR family members, such as PAR-3 and PAR-4, to ensure comprehensive receptor targeting. This balanced view could confirm whether the specificity of ATV and RSV toward PAR-2 is unique or part of a broader interaction with PAR family members.

Furthermore, future experiments could employ phosphoproteomics, pathway-specific inhibitors, or siRNA knockdown techniques to dissect the signalling networks modulated by statins at a more granular level. This would provide valuable insights into how PAR-2 inhibition translates into reduced cancer progression, offering a mechanistic basis for developing adjunctive therapies for CRC.

By building on the mechanistic foundations laid in this study, such future investigations could establish the full therapeutic potential of statins. This would bridge the current knowledge gap regarding chemo-resistance pathways and statin interactions with other therapeutic agents, contributing to personalized CRC treatment strategies.

Clinical translation and dosing strategy

The absence of *in vivo* studies or clinical data limits the immediate translational impact of the findings. While this study establishes a foundational understanding of statin effects on *PAR-2*, animal models and clinical trials are needed to validate these results *in vivo*. Determining optimal dosing regimens that maximize therapeutic efficacy while minimizing toxicity will be essential for integrating statins into CRC treatment protocols. Pharmacodynamic studies could further elucidate the dose-response relationships for ATV and RSV, guiding future personalized treatment strategies.

Comparison with similar research

The overexpression of PAR-2 in CRC has been well-documented in the literature. Darmoul *et al.* established that PAR-2 is highly expressed in colon cancer tissues and cell lines, contributing significantly to tumour progression and the pro-inflammatory microenvironment (101). Our study aligns with these findings, showing elevated PAR-2 expression in LPS-stimulated HT-29 and Caco-2

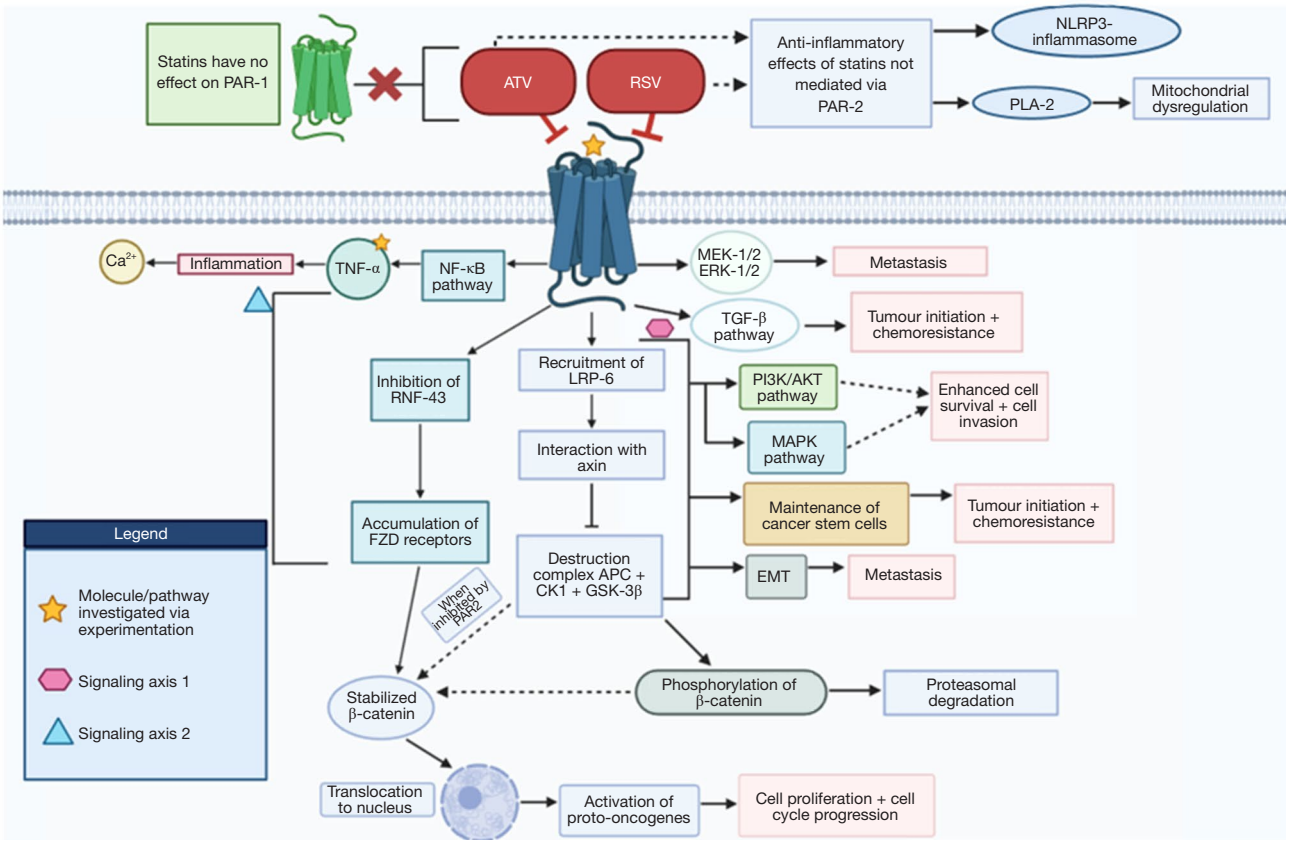

Figure 10 This figure illustrates the proposed mechanistic pathways by which ATV and RSV selectively inhibit *PAR-2* signaling in CRC cells, without affecting *PAR-1*. The inhibition of *PAR-2* by ATV and RSV disrupts multiple downstream signaling cascades associated with inflammation, tumor progression, and metastasis. The pathways investigated in this study are represented as follows: (I) *PAR-2* specificity: both ATV and RSV specifically target *PAR-2*, as indicated by the red blocks on the *PAR-2* receptor, while sparing *PAR-1*, which remains unaffected (top left of the figure). This specificity underscores the selective effect of statins on pro-inflammatory pathways without impacting vascular functions typically mediated by *PAR-1*. (II) Calcium-dependent inflammatory signaling: activation of *PAR-2* leads to calcium (Ca^{2+})-dependent pro-inflammatory signaling, resulting in the upregulation of TNF-α and activation of the NF-κB pathway, both of which are key drivers of inflammation in CRC (shown on the left side). (III) LRP-6 and Wnt/β-catenin pathway: *PAR-2* activation recruits *LRP-6* and interacts with *Axin*, leading to the accumulation of stabilized *β-catenin*, which translocates to the nucleus and activates proto-oncogenes, promoting cell proliferation and tumor progression. Statin-mediated inhibition of *PAR-2* disrupts this pathway, preventing *β-catenin* stabilization and subsequent oncogenic signaling (center). (IV) Inhibition of PI3K/Akt and MAPK pathways: PAR-2 signaling enhances the PI3K/Akt and MAPK pathways, which are involved in cell survival, EMT (epithelial-mesenchymal transition), cancer stem cell maintenance, and metastasis. ATV and RSV suppress these pathways by targeting *PAR-2*, thereby reducing CRC cell survival, chemoresistance, and metastatic potential (center and right side). (V) Anti-Inflammatory and non-PAR-2-mediated effects: statins also exhibit anti-inflammatory effects that are independent of *PAR-2*, including the inhibition of the *NLRP3* (NOD-, LRR- and Pyrin Domain-Containing Protein 3) inflammasome, *PLA2* activity, and mitochondrial dysregulation. These effects provide additional anti-inflammatory benefits that support CRC treatment (top right). (VI) Role of ERK and TGF-β pathway: Statin treatment inhibits *PAR-2*-mediated *ERK* and *TGF-β* signaling, which are implicated in metastasis, tumor initiation, and chemoresistance, further reducing the aggressive behavior of CRC cells. Molecules and pathways directly investigated in this study are marked with specific symbols for clarity. Signaling pathways are indicated with color-coded arrows to distinguish between two main signaling axes observed in *PAR-2*-mediated pathways. Overall, this figure integrates the molecular effects of statins on *PAR-2* signaling and its downstream pathways, emphasizing the therapeutic potential of ATV and RSV in attenuating *PAR-2*-mediated inflammatory and tumorigenic processes in colorectal cancer. ATV, atorvastatin; CRC, colorectal cancer; RSV, rosuvastatin.

cells, indicating that PAR-2 plays a critical role in CRC pathology. Interestingly, a recent study by Shah *et al.* showed that PAR-2 activation attenuates doxorubicin-induced cell death in HT29 cells, suggesting a role for PAR-2 in chemoresistance (55). This finding corroborates our observations and highlights the potential of PAR-2 modulation in enhancing chemotherapeutic efficacy. Furthermore, Quan *et al.* reported that PAR-2 inhibition enhanced the sensitivity of CRC cells to 5-FU and reduced EMT signalling (57). These studies collectively underscore the importance of PAR-2 in CRC progression and treatment resistance.

The anti-inflammatory effects of statins in CRC have been previously reported, but the mechanisms were not fully elucidated. A meta-analysis involving 52 studies and over 11 million individuals found that statin use reduced CRC risk by 20% in the general population, with even more pronounced effects—up to 60% risk reduction in individuals with IBD (P<0.001) (102). These findings suggest that statins may be particularly effective in modulating chronic inflammation, a key driver of CRC progression, especially in high-risk populations such as those with inflammation-related disorders. Additional evidence from a nationwide cohort study from Sweden confirmed that statins are associated with a 24% reduction in CRC incidence [adjusted hazard ratio (HR): 0.76] and a 44% reduction in CRC-related mortality (adjusted HR: 0.56). The benefit was most pronounced in patients using statins for five or more years [adjusted odds ratio (OR): 0.38], emphasizing the potential for long-term statin use to prevent CRC (24). Notably, the protective effects were primarily observed in patients with UC rather than Crohn's disease, suggesting a possible interaction between statin therapy and the inflammatory pathways specific to UC. Another meta-analysis comprising 40 studies similarly concluded that statin use was associated with a modest reduction in CRC risk. While the reduction was not statistically significant in randomized clinical trials, it achieved significance in observational studies, pointing to the potential for statins as chemopreventive agents (103). However, the precise mechanisms underlying these effects remained unclear, underscoring the need for more focused research on how statins interact with tumour biology, particularly through specific pathways such as PAR-2 signalling. Our findings address this gap by providing a potential molecular mechanism for the chemopreventive effects observed in previous research.

Stein *et al.* investigated the role of PAR-2 in calcium mobilization within cancer cells, particularly in triple-negative breast cancer (MDA-MB-231). Their study demonstrated that activation of PAR-2 by agonists, such as the SLIGKV-NH2 peptide, triggers significant intracellular calcium release, highlighting calcium as a key secondary messenger in cancer progression, migration, and inflammation, as corroborated by other studies detailed in Feldenzer's review, which provides additional insights into calcium's pivotal role in oncogenic signalling pathways (104,105). Inhibition of PAR-2 with synthetic indolactam V analogues significantly reduced calcium mobilization, reinforcing the link between PAR-2 activation and calcium flux-driven tumour migration and invasion. This underscores PAR-2 as a viable therapeutic target for blocking metastasis and inflammation. In complementary findings, Iablokov *et al.* further elucidated the impact of PAR-2 activation on calcium signalling and apoptosis in CRC cells. In HT-29 cells, PAR-2 activation leads to intracellular calcium influx through the activation of PI3K and MEK1/2 pathways, which are essential for cancer cell proliferation and survival (106). Elevated calcium levels were found to support cell survival by promoting the phosphorylation of BAD and MCL-1, two pro-survival proteins that stabilize mitochondrial integrity. Notably, when PAR-2 signalling was inhibited via siRNA, calcium influx was diminished, sensitizing the cells to apoptosis induced by TNF-α and IFN-γ, thereby underscoring the importance of PAR-2 in maintaining cancer cell viability. In the context of our study, the observed reduction in calcium fluorescence following treatment with ATV and RSV is consistent with the suppression of PAR-2-mediated calcium mobilization. As calcium influx is a key downstream effect of PAR-2 activation, our results suggest that ATV and RSV effectively inhibit PAR-2 signalling, thereby disrupting calcium-dependent inflammatory and survival pathways in CRC. This aligns with prior research, highlighting the potential of statins to impair tumour progression by targeting PAR-2 and attenuating calcium-driven tumorigenic behaviour, which may translate into effective therapeutic strategies for inflammation-associated cancers.

Apart from the PAR-2-mediated effects on intracellular calcium signalling observed in our study, statins may modulate calcium levels through additional independent mechanisms that warrant further investigation. For example, pravastatin has been shown to alleviate IL-6-induced calcium dysregulation in adult ventricular myocytes, suggesting that statins may restore calcium homeostasis under inflammatory conditions by mechanisms distinct from PAR-2 inhibition (107). Furthermore, statins are known

to inhibit inflammatory pathways such as the NLRP3 inflammasome and TLR pathways (108,109), both of which contribute to elevated intracellular calcium (110,111). By reducing the activation of these pathways, statins may indirectly limit calcium influx, thereby modulating inflammatory responses in CRC cells. Additionally, statins lower oxidative stress by decreasing the production of reactive oxygen species (ROS), which is another factor that influences calcium channel function and intracellular calcium balance (112). This reduction in oxidative stress could play a role in maintaining normal calcium signaling, preventing excess calcium-driven inflammation and tumour progression (113). These complementary pathways underscore the need for future research to investigate whether statins affect intracellular calcium independently of PAR-2, especially in the context of cancer, to better understand their full therapeutic potential.

Statins have been reported to modulate TNF-α levels. For instance, in hepatic inflammation models, RSV has been shown to reduce TNF-α expression through pathways unrelated to PAR-2 (114), thereby alleviating chronic inflammation and potentially preventing tumour progression. Similarly, statins have been found to inhibit TNF-α production in stimulated T-lymphocytes, involving the suppression of the NLRP3 inflammasome and TLR pathways (115), which are critical regulators of systemic inflammation. Furthermore, the inhibition of the TBK1-IRF3-IL-33 signalling pathway by statins, which also leads to reduced TNF-α levels (116,117), underscores their broad anti-inflammatory capabilities, where attenuation of TNF-α occurs through mechanisms independent of PAR-2. In our study, while we similarly observe a reduction in TNF-α levels following statin treatment, this is accompanied by a concomitant reduction in PAR-2 expression, suggesting a PAR-2-dependent mechanism. This finding indicates that statins may directly inhibit PAR-2 expression in CRC cells, thereby attenuating TNF-α secretion. This mechanism is consistent with our observations in pro-inflammatory chondrocytes, where siRNA-mediated knockdown of PAR-2 resulted in a significant reduction of TNF-α levels, though it did not completely eliminate TNF-α production (118), pointing to the existence of both PAR-2-dependent and independent mechanisms in the modulation of TNF-α levels. Thus, our study introduces a new dimension to the understanding of statins' anti-inflammatory actions by highlighting a PAR-2-dependent pathway, particularly relevant in the context of CRC inflammation, where PAR-2 signalling appears to play a crucial role in modulating TNF-α secretion and downstream inflammatory pathways. Further investigation is needed to clarify how both PAR-2-dependent and independent mechanisms contribute to the overall anti-inflammatory and anti-cancer effects of statins in CRC.

Explanation of findings

Our study demonstrates that both ATV and RSV selectively inhibit PAR-2 expression and related signalling in CRC cell lines (HT-29 and Caco-2) stimulated by LPS, without altering PAR-1 expression. This selectivity underscores the targeted action of these statins in reducing inflammation and tumour progression through PAR-2-specific pathways.

The selective inhibition of PAR-2 by ATV and RSV, while sparing PAR-1, emphasizes the differential signalling mechanisms and functional roles of these receptors in CRC. As detailed in the *Introduction*, PAR-2 activation is closely linked to key oncogenic pathways, including NF-κB, PI3K/Akt, MAPK/ERK, COX-2, Rho/Rac signalling, and phospholipase A2 (PLA2) activity, all of which contribute to inflammation, cell proliferation, and tumour progression (*Figure 10*). By downregulating PAR-2 expression, ATV and RSV are likely to inhibit these downstream pathways, thereby attenuating pro-inflammatory signalling, reducing tumour growth, and potentially preventing metastasis.

The mechanisms by which statins selectively downregulate PAR-2 in HT-29 and Caco-2 cell lines can be linked to their effects on the mevalonate pathway, which inhibits isoprenoid synthesis required for GTPase prenylation. This disrupts PAR-2-mediated signaling pathways that are heavily dependent on small GTPases like Rho and Rac (119). In human umbilical vein endothelial cells (HUVECs), statins were found to prevent tissue factor (TF) induction by both PAR-1 and PAR-2 by reducing cholesterol content in caveolin-enriched membrane microdomains, which are essential for maintaining receptor localization and function (120). Specifically, statins induced the relocation of PAR-1 from these microdomains to high-density membrane fractions, impairing TF induction and PAR-1's signaling capacity (120). Additionally, statins selectively decreased PAR-2 mRNA expression but not PAR-1 mRNA levels, further contributing to the inhibition of PAR-2-mediated TF induction (120). Importantly, this effect was shown to be specific to the inhibition of HMG-CoA reductase, as it was reversed by the addition of mevalonate (120). This mechanism aligns with the effect observed in CRC cells, where the disruption of lipid rafts

by statins (121), particularly RSV, affects PAR-2 more than PAR-1, potentially due to PAR-2's greater reliance on these microdomains for signaling in cancer cells. Statins in the HUVEC study also impaired ERK1/2 activation, which is essential for TF induction through both PAR-1 and PAR-2 pathways, further highlighting the significant role of statin-mediated membrane alterations in suppressing inflammatory and pro-thrombotic signaling pathways (120).

Conversely, PAR-1, which primarily mediates thrombin signaling in vascular processes, relies less on GTPase-dependent pathways (122), making it less susceptible to statin-induced modulation in CRC cells. The difference in response between HUVECs and CRC cells may also stem from distinct cellular contexts and membrane lipid compositions, as well as differences in mRNA regulation; in HUVECs, PAR-1 mRNA levels are not affected, whereas in CRC cell lines, statins do not impact PAR-1 signaling due to the receptor's reduced reliance on lipid rafts and isoprenoid-dependent pathways (120).

PAR-2 downregulation by statins could also impact the TME in CRC. The TME, characterized by a pro-inflammatory milieu, supports tumour growth, immune evasion, and metastasis, with PAR-2 playing a central role in orchestrating these processes through cytokine release, angiogenesis, and stromal remodelling (122). By inhibiting PAR-2, ATV and RSV may reduce pro-tumorigenic signalling within the TME, which could enhance the immune response against cancer cells, decrease stromal support for tumour growth (121), and limit metastasis (123). This reduction in TME-driven inflammation and support potentially translates to better control of tumour progression.

Additionally, PAR-2 activation has been implicated in drug resistance mechanisms, as it contributes to the reduced sensitivity of cancer cells to chemotherapeutic agents by enhancing survival signalling pathways and promoting the expression of drug efflux pumps (55,57,124,125). The downregulation of PAR-2 by statins could sensitize CRC cells to chemotherapy by lowering the activation of pathways such as PI3K/Akt and NF-κB, which are involved in cell survival and resistance to apoptosis induced by drugs like doxorubicin (126). Therefore, the combination of statins with existing chemotherapeutic agents could offer a synergistic approach, improving the efficacy of treatment through PAR-2 inhibition and increased drug sensitivity.

The downregulation of TNF-α and calcium mobilization observed in our study can also be attributed to the selective inhibition of PAR-2 by statins (*Figure 10*). PAR-2 activation is known to trigger Gq protein-coupled signalling, which leads to calcium release from intracellular stores (127), and promotes NF-κB activation, driving TNF-α secretion. By downregulating PAR-2, ATV and RSV effectively reduce calcium signalling and TNF-α production, further supporting their role in attenuating inflammation and tumour progression through this specific pathway.

Our study builds on the role of PAR-2 as a central mediator in inflammatory processes within endothelial and cancerous cells, contributing to a range of pathological conditions, including vascular inflammation and CRC progression. The findings from Al-Ani *et al.* (128) significantly enrich our understanding of the pathways by which PAR-2 activation impacts cellular signaling and highlight the potential for statins to attenuate these effects. In endothelial cells, PAR-2 activation, through specific agonist peptides or by FXa, has been shown to increase soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) release, a factor implicated in endothelial damage. The mechanism of sVEGFR-1 release appears tightly regulated by several kinases and signaling pathways. Specifically, PAR-2-induced sVEGFR-1 release requires the activity of PKC isozymes, particularly PKCβ1 and PKCε, as well as Src family kinases. The Src kinases, when inhibited, significantly attenuate both sVEGFR-1 release and VEGFR-1 promoter activity, highlighting the importance of Src-dependent phosphorylation in this pathway. Moreover, the MAPK pathway, specifically through ERK-1/2 phosphorylation, is essential for PAR-2-mediated sVEGFR-1 release. The involvement of MEK-1/2, a critical upstream regulator of ERK-1/2, further underscores the significance of MAPK signaling in PAR-2-activated responses. This pathway convergence points to ERK-1/2 as a potential downstream target through which PAR-2 exerts its inflammatory effects, leading to elevated sVEGFR-1 levels. This finding has broader implications for other systems where ERK-1/2 activity is integral to the cellular response to inflammation and growth signals, including in CRC.

A particularly noteworthy mechanism in PAR-2 signaling, as highlighted by Al-Ani *et al.* (128), is the role of EGFR transactivation in sVEGFR-1 release. Upon PAR-2 activation, intracellular signaling mediated by PKC and Src promotes EGFR transactivation, which in turn amplifies downstream signaling cascades, including MAPK/ERK-1/2. The inhibition of EGFR with AG1478 results in the complete blockade of sVEGFR-1 release and ERK-1/2 phosphorylation, emphasizing EGFR's central role in PAR-2-mediated responses. This transactivation mechanism

is particularly relevant to cancer, where EGFR and ERK signaling are commonly upregulated and associated with tumor growth and survival. An additional regulatory axis involving heme oxygenase-1 (HO-1) and carbon monoxide (CO) provides further complexity to the PAR-2 pathway. HO-1 and its metabolic product CO downregulate sVEGFR-1 release through suppression of PAR-2 signaling. Statins, by upregulating HO-1 expression, indirectly reduce PAR-2-mediated sVEGFR-1 release, indicating a possible mechanism for their anti-inflammatory effects. The application of simvastatin in Al-Ani's study significantly reduced PAR-2-induced sVEGFR-1 release (128), aligning with our study's focus on statin-mediated PAR-2 inhibition. This suggests that statins might not only disrupt isoprenoid synthesis necessary for GTPase-dependent signaling but also reduce PAR-2 activation through pathways involving HO-1 and CO.

In our study, we observed that ATV and RSV selectively downregulated PAR-2 expression in CRC cells, with downstream inhibition of inflammatory cytokines. This selective inhibition of PAR-2, while sparing PAR-1, aligns with findings that statins can disrupt lipid rafts essential for PAR-2 localization and function. Furthermore, Al-Ani's findings (128), support our hypothesis that statins reduce PAR-2-related signaling by mechanisms extending beyond direct receptor interaction. The statin-mediated upregulation of HO-1 and suppression of ERK-1/2 activation provide indirect yet effective pathways for reducing PAR-2-driven inflammatory responses. Therefore, the anti-inflammatory effects of statins could suppress PAR-2 indirectly.

Apart from PAR-2-specific effects, statins are known to exert broader anti-inflammatory effects through mechanisms such as inhibition of the NLRP3 inflammasome (129), reduction of PLA2 activity (130), and attenuation of mitochondrial function (131). These pathways indirectly impact intracellular calcium levels by modulating cellular stress responses, further contributing to the anti-inflammatory effects observed in our study.

Implications and actions needed

The findings from this study indicate that ATV and RSV specifically inhibit PAR-2 expression and related inflammatory signaling in CRC cell lines, while sparing PAR-1. This selective modulation has significant implications for managing inflammation-driven CRC, particularly through targeting PAR-2-related pathways. Below are the detailed implications and suggested actions:

Adjunctive therapy potential in CRC

The selective downregulation of PAR-2 by statins, as demonstrated in this study, adds a novel dimension to their well-established anti-inflammatory properties. This suggests that statins could be effectively incorporated as adjunctive treatments in CRC management to reduce inflammation and tumor growth while potentially enhancing the efficacy of existing chemotherapies.

Modulation of TME

The TME plays a crucial role in supporting cancer progression and treatment resistance. By specifically targeting PAR-2, statins have the potential to modulate the TME by reducing pro-inflammatory cytokine release, limiting stromal support for tumor growth, and enhancing antitumor immune responses. This broader therapeutic role for statins warrants further investigation in *in vivo* models to better understand their impact on the TME dynamics.

Enhanced drug sensitivity

The study also highlights that PAR-2 downregulation by statins may increase the sensitivity of CRC cells to chemotherapeutic agents, as PAR-2 has been implicated in mechanisms of drug resistance, including survival signaling and the upregulation of drug efflux pumps. The potential to improve chemotherapeutic efficacy through statin co-treatment should be further explored through combination therapy studies.

Broader mechanistic insights into statin action

These findings underscore that beyond their cholesterol-lowering effects, statins can selectively inhibit specific GPCR-mediated signaling pathways, such as those driven by PAR-2. This raises the possibility of broader therapeutic implications, as the modulation of GPCR signaling may extend beyond CRC to other inflammation-driven cancers, suggesting a need for further mechanistic studies.

Future preclinical and clinical investigations

To translate these promising findings into clinical applications, it is imperative to conduct comprehensive *in vivo* studies to evaluate the effects of statins on PAR-2 expression, tumor progression, and survival in CRC models. Additionally, clinical trials should focus on identifying optimal dosing strategies for combining statins with standard therapies, aiming to enhance safety, efficacy, and

the reduction of inflammation-driven CRC progression.

Recommended actions

- Preclinical validation: utilize animal models to further evaluate the impact of statins on PAR-2 expression, inflammatory signaling, and TME modulation in CRC.
- Clinical trials: design clinical trials to assess the efficacy of statins as adjunctive therapies in CRC, with a focus on PAR-2-specific outcomes, chemoresistance, and tumor progression.
- Mechanistic studies: investigate the broader effects of statins on GPCR signaling and associated pathways, aiming to uncover potential therapeutic benefits in other inflammation-driven cancers.
- TME-focused research: examine the effects of statins on the TME, emphasizing immune modulation, stromal interactions, and overall impact on CRC progression.

Conclusions

This study demonstrates the selective inhibition of PAR-2 expression and related signaling pathways by ATV and RSV in LPS-stimulated CRC cell lines. By targeting PAR-2, both statins significantly attenuate inflammatory responses, TNF-α secretion, and calcium mobilization, while sparing PAR-1 expression. This specificity not only underscores their therapeutic potential in controlling inflammation-driven CRC, but also differentiates them from other treatment strategies that often affect broader signaling networks, including those critical for vascular homeostasis mediated by PAR-1.

The selective downregulation of PAR-2 is mechanistically linked to the mevalonate pathway inhibition by statins, which disrupts isoprenoid synthesis and subsequently GTPase activation, crucial for PAR-2-mediated signaling. The observed reduction in calcium signaling, alongside diminished TNF-α secretion, suggests that statins not only suppress pro-inflammatory signaling but also mitigate the TME, thereby inhibiting tumor progression. This modulation of the TME, marked by decreased cytokine release, reduced stromal support, and enhanced immune responsiveness, further supports the potential of statins as adjunctive treatments in CRC.

Additionally, this study identifies a plausible mechanism by which statins could enhance chemotherapeutic efficacy by increasing drug sensitivity through PAR-2 inhibition. The suppression of survival signaling pathways, such as PI3K/Akt and NF-κB, highlights their role in overcoming drug resistance, a major challenge in cancer therapy. Given the broader anti-inflammatory actions of statins, including NLRP3 inflammasome inhibition, reduction of PLA2 activity, and modulation of mitochondrial function, their integration into CRC treatment regimens presents a promising multi-dimensional therapeutic approach.

While this study provides compelling *in vitro* evidence of the anti-inflammatory and potential anti-cancer effects of statins through PAR-2-specific pathways, it is critical to validate these findings in *in vivo* models and clinical trials. The translation of these results into clinical practice requires a deeper understanding of the optimal dosing strategies, long-term outcomes, and the broader implications of statin use in CRC management. Future research should focus on clarifying the complex interplay between PAR-2-specific signaling, the TME, and chemoresistance mechanisms, with an aim to establish statins as a standard adjunctive therapy in inflammation-driven CRC.

In summary, this study not only advances our understanding of statin action beyond cholesterol modulation but also paves the way for novel therapeutic strategies in managing inflammation-driven malignancies like CRC. The selective targeting of PAR-2 by statins represents a significant step toward more precise, safe, and effective cancer therapies, warranting further exploration and clinical application.

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Footnote

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References

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021;71:209-49.
2. Naidoo N, Bajwa G, Duvuru R, et al. Thanatogenomic Investigation of the Hydroxymethylome and Mitochondrial Genome of Cadaveric Cardiomyocytes: Proposal for a Proof-of-Concept Study. JMIR Res Protoc 2020;9:e17241.
3. Al Abdouli L, Dalmook H, Akram Abdo M, et al. Colorectal Cancer Risk Awareness and Screening Uptake among Adults in the United Arab Emirates. Asian Pac J Cancer Prev 2018;19:2343-9.
4. Alsaad LN, Sreedharan J. Practice of colorectal cancer screening in the United Arab Emirates and factors associated - a cross-sectional study. BMC Public Health 2023;23:2015.
5. Mamdouh H, Hussain HY, Ibrahim GM, et al. Prevalence and associated risk factors of overweight and obesity among adult population in Dubai: a population-based cross-sectional survey in Dubai, the United Arab Emirates. BMJ Open 2023;13:e062053.
6. Shen Y, Wang C, Ren Y, et al. A comprehensive look at the role of hyperlipidemia in promoting colorectal cancer liver metastasis. J Cancer 2018;9:2981-6.
7. Bray F, Laversanne M, Sung H, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2024;74:229-63.
8. Chen K, Guo J, Zhang T, et al. The Role of Dyslipidemia in Colitis-Associated Colorectal Cancer. J Oncol 2021;2021:6640384.
9. Qiang JK, Lipscombe LL, Lega IC. Association between diabetes, obesity, aging, and cancer: review of recent literature. Transl Cancer Res 2020;9:5743-59.
10. Rychter AM, Łykowska-Szuber L, Zawada A, et al. Why Does Obesity as an Inflammatory Condition Predispose to Colorectal Cancer? J Clin Med 2023;12:2451.
11. Zhong J, Guo J, Zhang X, et al. The remodeling roles of lipid metabolism in colorectal cancer cells and immune microenvironment. Oncol Res 2022;30:231-42.
12. Lucafò M, Curci D, Franzin M, et al. Inflammatory Bowel Disease and Risk of Colorectal Cancer: An Overview From Pathophysiology to Pharmacological Prevention. Front Pharmacol 2021;12:772101.
13. Freeman HJ. Colorectal cancer risk in Crohn's disease. World J Gastroenterol 2008;14:1810-1.
14. Li W, Li L, Yang H, et al. Unraveling the Role of TP53 in Colorectal Cancer Therapy: From Wild-Type Regulation to Mutant. Front Biosci (Landmark Ed) 2024;29:272.
15. Zhou Y, Kuang Y, Wang C, et al. Impact of KRAS mutation on the tumor microenvironment in colorectal cancer. Int J Biol Sci 2024;20:1947-64.
16. Farahani H, Mahmoudi T, Asadi A, et al. Insulin Resistance and Colorectal Cancer Risk: the Role of Elevated Plasma Resistin Levels. J Gastrointest Cancer 2020;51:478-83.
17. Giovannucci E, Harlan DM, Archer MC, et al. Diabetes and cancer: a consensus report. Diabetes Care 2010;33:1674-85.
18. Kaaks R, Lukanova A. Energy balance and cancer: the role of insulin and insulin-like growth factor-I. Proc Nutr Soc 2001;60:91-106.
19. Vetvicka D, Suhaj P, Olejar T, et al. Proteinase-activated Receptor 2: Springboard of Tumors. Anticancer Res 2024;44:1-12.
20. Jannati S, Patnaik R, Banerjee Y. Beyond Anticoagulation: A Comprehensive Review of Non-Vitamin K Oral Anticoagulants (NOACs) in Inflammation and Protease-Activated Receptor Signaling. Int J Mol Sci 2024;25:8727.

21. Macfarlane SR, Seatter MJ, Kanke T, et al. Proteinase-activated receptors. Pharmacol Rev 2001;53:245-82.
22. Lv J, Liu J, Chao G, et al. PARs in the inflammation-cancer transformation of CRC. Clin Transl Oncol 2023;25:1242-51.
23. Darmoul D, Gratio V, Devaud H, et al. Protease-activated receptor 2 in colon cancer: trypsin-induced MAPK phosphorylation and cell proliferation are mediated by epidermal growth factor receptor transactivation. J Biol Chem 2004;279:20927-34.
24. Sun J, Halfvarson J, Bergman D, et al. Statin use and risk of colorectal cancer in patients with inflammatory bowel disease. EClinicalMedicine 2023;63:102182.
25. Fernando EH, Gordon MH, Beck PL, et al. Inhibition of Intestinal Epithelial Wound Healing through Protease-Activated Receptor-2 Activation in Caco2 Cells. J Pharmacol Exp Ther 2018;367:382-92.
26. Ma Y, He L, Zhao X, et al. Protease activated receptor 2 signaling promotes self-renewal and metastasis in colorectal cancer through beta-catenin and periostin. Cancer Lett 2021;521:130-41.
27. Tsai CC, Chou YT, Fu HW. Protease-activated receptor 2 induces migration and promotes Slug-mediated epithelial-mesenchymal transition in lung adenocarcinoma cells. Biochim Biophys Acta Mol Cell Res 2019;1866:486-503.
28. Nag JK, Kancharla A, Maoz M, et al. Low-density lipoprotein receptor-related protein 6 is a novel coreceptor of protease-activated receptor-2 in the dynamics of cancer-associated β-catenin stabilization. Oncotarget 2017;8:38650-67.
29. Beagle B, Mi K, Johnson GV. Phosphorylation of PPP(S/T)P motif of the free LRP6 intracellular domain is not required to activate the Wnt/beta-catenin pathway and attenuate GSK3beta activity. J Cell Biochem 2009;108:886-95.
30. Stamos JL, Weis WI. The β-catenin destruction complex. Cold Spring Harb Perspect Biol 2013;5:a007898.
31. Zhang Y, Wang X. Targeting the Wnt/β-catenin signaling pathway in cancer. J Hematol Oncol 2020;13:165.
32. Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 1999;19:1-11.
33. Hinz M, Krappmann D, Eichten A, et al. NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol 1999;19:2690-8.
34. Jean-Charles PY, Kaur S, Shenoy SK. G Protein-Coupled Receptor Signaling Through β-Arrestin-Dependent Mechanisms. J Cardiovasc Pharmacol 2017;70:142-58.
35. Shishido A, Miyo M, Oishi K, et al. The Relationship between LRP6 and Wnt/β-Catenin Pathway in Colorectal and Esophageal Cancer. Life (Basel) 2023;13:615.
36. Jeong W, Jho EH. Regulation of the Low-Density Lipoprotein Receptor-Related Protein LRP6 and Its Association With Disease: Wnt/β-Catenin Signaling and Beyond. Front Cell Dev Biol 2021;9:714330.
37. Parker TW, Neufeld KL. APC controls Wnt-induced β-catenin destruction complex recruitment in human colonocytes. Sci Rep 2020;10:2957.
38. Ren Q, Chen J, Liu Y. LRP5 and LRP6 in Wnt Signaling: Similarity and Divergence. Front Cell Dev Biol 2021;9:670960.
39. Chen X, Zhang Y, Zhang P, et al. IGFBP2 drives epithelial-mesenchymal transition in hepatocellular carcinoma via activating the Wnt/β-catenin pathway. Infect Agent Cancer 2023;18:73.
40. Nag JK, Appasamy P, Sedley S, et al. RNF43 induces the turnover of protease-activated receptor 2 in colon cancer. FASEB J 2023;37:e22675.
41. Farnhammer F, Colozza G, Kim J. RNF43 and ZNRF3 in Wnt Signaling - A Master Regulator at the Membrane. Int J Stem Cells 2023;16:376-84.
42. Giannakis M, Hodis E, Jasmine Mu X, et al. RNF43 is frequently mutated in colorectal and endometrial cancers. Nat Genet 2014;46:1264-6.
43. Koo BK, van Es JH, van den Born M, et al. Porcupine inhibitor suppresses paracrine Wnt-driven growth of Rnf43;Znrf3-mutant neoplasia. Proc Natl Acad Sci U S A 2015;112:7548-50.
44. Rohani MG, DiJulio DH, An JY, et al. PAR1- and PAR2-induced innate immune markers are negatively regulated by PI3K/Akt signaling pathway in oral keratinocytes. BMC Immunol 2010;11:53.
45. Li X, Wu Y, Tian T. TGF-β Signaling in Metastatic Colorectal Cancer (mCRC): From Underlying Mechanism to Potential Applications in Clinical Development. Int J Mol Sci 2022;23:14436.
46. Ungefroren H, Witte D, Rauch BH, et al. Proteinase-Activated Receptor 2 May Drive Cancer Progression by Facilitating TGF-β Signaling. Int J Mol Sci 2017;18:2494.
47. Ungefroren H, Witte D, Fiedler C, et al. The Role of PAR2 in TGF-β1-Induced ERK Activation and Cell Motility. Int J Mol Sci 2017;18:2776.
48. Zhang M, Zhang YY, Chen Y, et al. TGF-β Signaling and Resistance to Cancer Therapy. Front Cell Dev Biol

2021;9:786728.
49. Mohammad MA, Greenman J, Maraveyas A, et al. Activation of PAR2 by tissue factor induces the release of the PTEN from MAGI proteins and regulates PTEN and Akt activities. Sci Rep 2020;10:20908.
50. Nasri I, Bonnet D, Zwarycz B, et al. PAR2-dependent activation of GSK3β regulates the survival of colon stem/progenitor cells. Am J Physiol Gastrointest Liver Physiol 2016;311:G221-36.
51. Johnson JJ, Miller DL, Jiang R, et al. Protease-activated Receptor-2 (PAR-2)-mediated Nf-κB Activation Suppresses Inflammation-associated Tumor Suppressor MicroRNAs in Oral Squamous Cell Carcinoma. J Biol Chem 2016;291:6936-45.
52. Shanshan H, Lan X, Xia L, et al. Inhibition of protease-activated receptor-2 induces apoptosis in cervical cancer by inhibiting signal transducer and activator of transcription-3 signaling. J Int Med Res 2019;47:1330-8.
53. Mo JS, Yu FX, Gong R, et al. Regulation of the Hippo-YAP pathway by protease-activated receptors (PARs). Genes Dev 2012;26:2138-43.
54. Chang JH, Park JM, Kim SW, et al. Expression of protease activated receptor-2 in human colorectal cancer and its association with tumor progression. Dis Colon Rectum 2010;53:1202-8.
55. Shah H, Hill TA, Lim J, et al. Protease-activated receptor 2 attenuates doxorubicin-induced apoptosis in colon cancer cells. J Cell Commun Signal 2023;17:1293-307.
56. Janani B, Vijayakumar M, Priya K, et al. EGFR-Based Targeted Therapy for Colorectal Cancer-Promises and Challenges. Vaccines (Basel) 2022;10:499.
57. Quan Q, Zhong F, Wang X, et al. PAR2 Inhibition Enhanced the Sensitivity of Colorectal Cancer Cells to 5-FU and Reduced EMT Signaling. Oncol Res. 2019;27:779-88.
58. Emran TB, Shahriar A, Mahmud AR, et al. Multidrug Resistance in Cancer: Understanding Molecular Mechanisms, Immunoprevention and Therapeutic Approaches. Front Oncol 2022;12:891652.
59. Malhi M, Norris MJ, Duan W, et al. Statin-mediated disruption of Rho GTPase prenylation and activity inhibits respiratory syncytial virus infection. Commun Biol 2021;4:1239.
60. El Khoury M, Naim HY. Lipid rafts disruption by statins negatively impacts the interaction between SARS-CoV-2 S1 subunit and ACE2 in intestinal epithelial cells. Front Microbiol 2023;14:1335458.
61. Vona R, Iessi E, Matarrese P. Role of Cholesterol and Lipid Rafts in Cancer Signaling: A Promising Therapeutic Opportunity? Front Cell Dev Biol 2021;9:622908.
62. Tripathi S, Gupta E, Galande S. Statins as anti-tumor agents: A paradigm for repurposed drugs. Cancer Rep (Hoboken) 2024;7:e2078.
63. Jia X, Lu Y, Xu Z, et al. Impact of statin use on breast cancer recurrence and mortality before and after diagnosis: a systematic review and meta-analysis. Front Oncol 2023;13:1256747.
64. Amin F, Fathi F, Reiner Ž, et al. The role of statins in lung cancer. Arch Med Sci 2022;18:141-52.
65. Chen TS, Liu HY, Chang YL, et al. Association between Statin Use and Clinical Outcomes in Patients with De Novo Metastatic Prostate Cancer: A Propensity Score-weighted Analysis. World J Mens Health 2024;42:630-7.
66. Dobrzycka M, Spychalski P, Łachiński AJ, et al. Statins and Colorectal Cancer - A Systematic Review. Exp Clin Endocrinol Diabetes 2020;128:255-62.
67. Pikoulis E, Margonis GA, Angelou A, et al. Statins in the chemoprevention of colorectal cancer in established animal models of sporadic and colitis-associated cancer. Eur J Cancer Prev 2016;25:102-8.
68. Jiang W, Hu JW, He XR, et al. Statins: a repurposed drug to fight cancer. J Exp Clin Cancer Res 2021;40:241.
69. Ouahoud S, Jacobs RJ, Peppelenbosch MP, et al. Kinome-wide analysis of the effect of statins in colorectal cancer. Br J Cancer 2021;124:1978-87.
70. Cai WS, Shen F, Li JL, et al. Activated protease receptor-2 induces GATA6 expression to promote survival in irradiated colon cancer cells. Arch Biochem Biophys 2014;555-556:28-32.
71. Žilinskas J, Stukas D, Jasukaitienė A, et al. Assessing the Therapeutic Impacts of HAMLET and FOLFOX on BRAF-Mutated Colorectal Cancer: A Study of Cancer Cell Survival and Mitochondrial Dynamics In Vitro and Ex Vivo. Medicina (Kaunas) 2024;60:142.
72. Patnaik R, Jannati S, Sivani BM, et al. Efficient Generation of Chondrocytes From Bone Marrow-Derived Mesenchymal Stem Cells in a 3D Culture System: Protocol for a Practical Model for Assessing Anti-Inflammatory Therapies. JMIR Res Protoc 2023;12:e42964.
73. Mastropietro G, Tiscornia I, Perelmuter K, et al. HT-29 and Caco-2 reporter cell lines for functional studies of nuclear factor kappa B activation. Mediators Inflamm 2015;2015:860534.
74. Cario E, Rosenberg IM, Brandwein SL, et al. Lipopolysaccharide activates distinct signaling pathways in

intestinal epithelial cell lines expressing Toll-like receptors. J Immunol 2000;164:966-72.
75. Beutler B. Endotoxin, toll-like receptor 4, and the afferent limb of innate immunity. Curr Opin Microbiol 2000;3:23-8.
76. Ronaldson PT, Brzica H, Abdullahi W, et al. Transport Properties of Statins by Organic Anion Transporting Polypeptide 1A2 and Regulation by Transforming Growth Factor-β Signaling in Human Endothelial Cells. J Pharmacol Exp Ther 2021;376:148-60.
77. Khatiwada N, Hong Z. Potential Benefits and Risks Associated with the Use of Statins. Pharmaceutics 2024;16:214.
78. Han X, Nieman MT, Kerlin BA. Protease-activated receptors: An illustrated review. Res Pract Thromb Haemost 2021;5:17-26.
79. Ramey-Ward AN, Walthall HP, Smith S, et al. Human keratin matrices promote wound healing by modulating skin cell expression of cytokines and growth factors. Wound Repair Regen 2024;32:257-67.
80. Reches G, Piran R. Par2-mediated responses in inflammation and regeneration: choosing between repair and damage. Inflamm Regen 2024;44:26.
81. Macfarlane SR, Sloss CM, Cameron P, et al. The role of intracellular Ca2+ in the regulation of proteinase-activated receptor-2 mediated nuclear factor kappa B signalling in keratinocytes. Br J Pharmacol 2005;145:535-44.
82. Gee KR, Brown KA, Chen WN, et al. Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. Cell Calcium 2000;27:97-106.
83. Anderle P, Niederer E, Rubas W, et al. P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels. J Pharm Sci 1998;87:757-62.
84. Climent E, Benaiges D, Pedro-Botet J. Hydrophilic or Lipophilic Statins? Front Cardiovasc Med 2021;8:687585.
85. Capozzi A, Manganelli V, Riitano G, et al. Advances in the Pathophysiology of Thrombosis in Antiphospholipid Syndrome: Molecular Mechanisms and Signaling through Lipid Rafts. J Clin Med 2023;12:891.
86. Sane R, Cheung KWK, Kovács P, et al. Calibrating the In Vitro-In Vivo Correlation for OATP-Mediated Drug-Drug Interactions with Rosuvastatin Using Static and PBPK Models. Drug Metab Dispos 2020;48:1264-70.
87. Gheytanchi E, Naseri M, Karimi-Busheri F, et al. Morphological and molecular characteristics of spheroid formation in HT-29 and Caco-2 colorectal cancer cell lines. Cancer Cell Int 2021;21:204.
88. Ataee R, Ajdary S, Zarrindast M, et al. Anti-mitogenic and apoptotic effects of 5-HT1B receptor antagonist on HT29 colorectal cancer cell line. J Cancer Res Clin Oncol 2010;136:1461-9.
89. Verhoeckx KCotter PLópez-Expósito I, et al. 2015.
90. Grimsey NJ, Trejo J. Integration of endothelial protease-activated receptor-1 inflammatory signaling by ubiquitin. Curr Opin Hematol 2016;23:274-9.
91. Guo XW, Lei RE, Zhou QN, et al. Tumor microenvironment characterization in colorectal cancer to identify prognostic and immunotherapy genes signature. BMC Cancer 2023;23:773.
92. Sieminska I, Baran J. Myeloid-Derived Suppressor Cells in Colorectal Cancer. Front Immunol 2020;11:1526.
93. Strating E, Verhagen MP, Wensink E, et al. Co-cultures of colon cancer cells and cancer-associated fibroblasts recapitulate the aggressive features of mesenchymal-like colon cancer. Front Immunol 2023;14:1053920.
94. Sayed IM, El-Hafeez AAA, Maity PP, et al. Modeling colorectal cancers using multidimensional organoids. Adv Cancer Res 2021;151:345-83.
95. Liu X, Xin Z, Wang K. Patient-derived xenograft model in colorectal cancer basic and translational research. Animal Model Exp Med 2023;6:26-40.
96. Hassin O, Nataraj NB, Shreberk-Shaked M, et al. Different hotspot p53 mutants exert distinct phenotypes and predict outcome of colorectal cancer patients. Nat Commun 2022;13:2800.
97. Lopez-Escalera S, Wellejus A. Evaluation of Caco-2 and human intestinal epithelial cells as in vitro models of colonic and small intestinal integrity. Biochem Biophys Rep 2022;31:101314.
98. Ahmed D, Eide PW, Eilertsen IA, et al. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2013;2:e71.
99. Glaviano A, Foo ASC, Lam HY, et al. PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. Mol Cancer 2023;22:138.
100. Zhang Z, Richmond A, Yan C. Immunomodulatory Properties of PI3K/AKT/mTOR and MAPK/MEK/ERK Inhibition Augment Response to Immune Checkpoint Blockade in Melanoma and Triple-Negative Breast Cancer. Int J Mol Sci 2022;23:7353.
101. Darmoul D, Gratio V, Devaud H, et al. Activation of proteinase-activated receptor 1 promotes human colon cancer cell proliferation through epidermal growth factor receptor transactivation. Mol Cancer Res 2004;2:514-22.
102. Rodríguez-Miguel A, Fernández-Antón E, Barreira-

Hernández D, et al. Statins and Colorectal Cancer Risk: A Population-Based Case-Control Study and Synthesis of the Epidemiological Evidence. J Clin Med 2022;11:1528.
103. Zhang JY, Wu QF, Wan Y, et al. Protective role of hydrogen-rich water on aspirin-induced gastric mucosal damage in rats. World J Gastroenterol 2014;20:1614-22.
104. Stein J, Stahn S, Neudörfl JM, et al. Synthetic Indolactam V Analogues as Inhibitors of PAR2-Induced Calcium Mobilization in Triple-Negative Breast Cancer Cells. ChemMedChem 2018;13:147-54.
105. Feldenzer KL, Sarno J. Hypercalcemia of Malignancy. J Adv Pract Oncol 2018;9:496-504.
106. Iablokov V, Hirota CL, Peplowski MA, et al. Proteinase-activated receptor 2 (PAR2) decreases apoptosis in colonic epithelial cells. J Biol Chem 2014;289:34366-34377.
107. Zuo S, Li L, Jiang L, et al. Pravastatin alleviates intracellular calcium dysregulation induced by Interleukin-6 via the mitochondrial ROS pathway in adult ventricular myocytes. J Pharmacol Sci 2020;143:141-7.
108. Koushki K, Shahbaz SK, Mashayekhi K, et al. Anti-inflammatory Action of Statins in Cardiovascular Disease: the Role of Inflammasome and Toll-Like Receptor Pathways. Clin Rev Allergy Immunol 2021;60:175-99.
109. Antonopoulos AS, Margaritis M, Lee R, et al. Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials. Curr Pharm Des 2012;18:1519-30.
110. Rossol M, Pierer M, Raulien N, et al. Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. Nat Commun 2012;3:1329.
111. Park KS, Kim SH, Das A, et al. TLR3-/4-Priming Differentially Promotes Ca(2+) Signaling and Cytokine Expression and Ca(2+)-Dependently Augments Cytokine Release in hMSCs. Sci Rep 2016;6:23103.
112. Kim SW, Kang HJ, Jhon M, et al. Statins and Inflammation: New Therapeutic Opportunities in Psychiatry. Front Psychiatry 2019;10:103.
113. Görlach A, Bertram K, Hudecova S, et al. Calcium and ROS: A mutual interplay. Redox Biol 2015;6:260-71.
114. Wang J, Liu C, Hu R, et al. Statin therapy: a potential adjuvant to immunotherapies in hepatocellular carcinoma. Front Pharmacol 2024;15:1324140.
115. Chen D, Sui L, Chen C, et al. Atorvastatin suppresses NLRP3 inflammasome activation in intracerebral hemorrhage via TLR4- and MyD88-dependent pathways. Aging (Albany NY) 2022;14:462-76.
116. Park JH, Mortaja M, Son HG, et al. Statin prevents cancer development in chronic inflammation by blocking interleukin 33 expression. Nat Commun 2024;15:4099.
117. Taranto D, Kloosterman DJ, Akkari L. Macrophages and T cells in metabolic disorder-associated cancers. Nat Rev Cancer 2024;24:744-67.
118. Patnaik R, Riaz S, Sivani BM, et al. Evaluating the potential of Vitamin D and curcumin to alleviate inflammation and mitigate the progression of osteoarthritis through their effects on human chondrocytes: A proof-of-concept investigation. PLoS One 2023;18:e0290739.
119. Jeong A, Suazo KF, Wood WG, et al. Isoprenoids and protein prenylation: implications in the pathogenesis and therapeutic intervention of Alzheimer's disease. Crit Rev Biochem Mol Biol 2018;53:279-310.
120. Banfi C, Brioschi M, Lento S, et al. Statins prevent tissue factor induction by protease-activated receptors 1 and 2 in human umbilical vein endothelial cells in vitro. J Thromb Haemost 2011;9:1608-19.
121. Xu M, Zhang T, Xia R, et al. Targeting the tumor stroma for cancer therapy. Mol Cancer 2022;21:208.
122. Schaffner F, Ruf W. Tissue factor and PAR2 signaling in the tumor microenvironment. Arterioscler Thromb Vasc Biol 2009;29:1999-2004.
123. Neophytou CM, Panagi M, Stylianopoulos T, et al. The Role of Tumor Microenvironment in Cancer Metastasis: Molecular Mechanisms and Therapeutic Opportunities. Cancers (Basel) 2021;13:2053.
124. Huo H, Feng Y, Tang Q. Inhibition of proteinase-activated receptor 2 (PAR2) decreased the malignant progression of lung cancer cells and increased the sensitivity to chemotherapy. Cancer Chemother Pharmacol. 2024;93:397-410. Erratum in: Cancer Chemother Pharmacol 2024;94:489.
125. Jiang Y, Zhuo X, Wu Y, et al. PAR2 blockade reverses osimertinib resistance in non-small-cell lung cancer cells via attenuating ERK-mediated EMT and PD-L1 expression. Biochim Biophys Acta Mol Cell Res 2022;1869:119144.
126. Cao Z, Liao Q, Su M, et al. AKT and ERK dual inhibitors: The way forward? Cancer Lett 2019;459:30-40.
127. Avet C, Sturino C, Grastilleur S, et al. The PAR2 inhibitor I-287 selectively targets Gαq and Gα12/13 signaling and has anti-inflammatory effects. Commun Biol 2020;3:719.
128. Al-Ani B, Hewett PW, Cudmore MJ, et al. Activation of proteinase-activated receptor 2 stimulates soluble vascular endothelial growth factor receptor 1 release via epidermal growth factor receptor transactivation in endothelial cells. Hypertension 2010;55:689-97.

129. Wang S, Xie X, Lei T, et al. Statins Attenuate Activation of the NLRP3 Inflammasome by Oxidized LDL or TNFα in Vascular Endothelial Cells through a PXR-Dependent Mechanism. Mol Pharmacol 2017;92:256-64.
130. Braun LT, Davidson MH. Lp-PLA2: A new target for statin therapy. Curr Atheroscler Rep 2010;12:29-33.
131. Mollazadeh H, Tavana E, Fanni G, et al. Effects of statins on mitochondrial pathways. J Cachexia Sarcopenia Muscle 2021;12:237-51.

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