Journal of Molecular Science

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 1005-1012
DoI-10.004687/1000-9035.2025.135

Journal of Molecular Science
www.jmolecularsci.com ISSN:1000-9035

SK1281, a Novel Peptide-Based IDO1 Inhibitor, Suppresses Tryptophan Metabolism and Induces Apoptosis in Colorectal Cancer Cells

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Article Information
Received: 07-10-2025
Revised: 25-10-2025
Accepted: 17-11-2025
Published: 13-12-2025

Keywords
Colorectal cancer; IDO1 inhibition; Immunometabolism; Kynurenine pathway; Peptide inhibitor; SK1281; Tryptophan catabolism; Apoptosis; Molecular docking

ABSTRACT
Indoleamine 2,3-dioxygenase 1 (IDO1) is a key metabolic immune checkpoint that degrades L-tryptophan into kynurenine, leading to strong suppression of antitumor immunity in colorectal cancer (CRC). Elevated IDO1 activity promotes tumor cell survival, immune evasion, and resistance to immunotherapy, highlighting IDO1 inhibition as a promising therapeutic strategy. In this study, we developed and evaluated a novel peptide-based compound, SK1281 (HFAVKHAVWAHVWHKAKSR), rationally designed with amphipathic and tryptophan-rich features to enhance cellular penetration and active-site binding. SK1281 demonstrated potent and dose-dependent inhibition of recombinant human IDO1 enzyme, achieving high-level activity comparable to the reference inhibitor IDO5L. In IFN-γ–stimulated colorectal cancer cell lines (HT29 and HCT116), SK1281 significantly suppressed cellular IDO1 activity, reversing tryptophan catabolism-driven metabolic dysregulation without inducing nonspecific toxicity. Furthermore, SK1281 triggered a marked increase in apoptotic cell death in a concentration-dependent manner, indicating that IDO1 blockade effectively reinstates tumor-suppressive pathways. Molecular docking and structural modeling revealed a stable binding orientation of SK1281 within the IDO1 catalytic pocket through hydrogen bonding to Ser167 and His346 and hydrophobic interactions adjacent to the heme cofactor, supporting a mechanistic basis for competitive enzyme inhibition. Collectively, these findings identify SK1281 as a promising next-generation IDO1 inhibitor with dual immunometabolic and direct anticancer effects, supporting its advancement toward preclinical development as a potential therapeutic candidate for colorectal cancer.

1. INTRODUCTION:

Colorectal cancer (CRC) remains one of the most prevalent and lethal malignancies worldwide, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths annually (Sung et al., 2021). Although advances in chemotherapeutic and targeted agents have contributed to improved outcomes, many patients develop immune evasion and therapeutic resistance, leading to disease progression and poor survival (de Visser et al., 2006; Ganesh et al., 2019). This has driven major interest toward immunometabolic pathways that shape tumor–immune interactions within the tumor microenvironment (TME).

Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-containing dioxygenase and the first rate-limiting enzyme in the kynurenine pathway of tryptophan metabolism (Munn & Mellor, 2007a). IDO1 catalyzes the oxidative conversion of L-tryptophan into N-formylkynurenine, ultimately populating immunoregulatory metabolites such as kynurenine, which suppresses effector T-cell activity and drives(Platten et al., 2021; Prendergast et al., 2017; Uyttenhove et al., 2003) regulatory T-cell (Treg) differentiation. IDO1 expression is strongly

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induced by inflammatory cytokines, particularly interferon-gamma (IFN-γ), which paradoxically enables tumors to hijack immune signaling to establish metabolic immune tolerance rather than destruction(Pallotta et al., 2022) . Elevated IDO1 activity results in local tryptophan starvation, T-cell cycle arrest, activation of the aryl hydrocarbon receptor (AhR), and generation of an immune-privileged microenvironment that fosters tumor survival, metastasis, and resistance to immunotherapy(Opitz et al., 2011)

High IDO1 expression has been detected in numerous malignancies, including CRC, and correlates with adverse clinical outcomes such as advanced stage, lymph node involvement, metastasis, and reduced overall survival (Yin et al., 2025). Due to its ability to strongly regulate antitumor immunity, IDO1 is now recognized as a metabolic immune checkpoint and a compelling therapeutic target complementary to PD-1/PD-L1 and CTLA-4 blockade (Charehjoo et al., 2023).

Several IDO1 inhibitors have entered clinical evaluation, including epacadostat and linrodostat. While strong preclinical outcomes were reported, clinical trials combining IDO1 inhibition with checkpoint blockade failed to yield expected benefits, largely due to insufficient intratumoral inhibition, compensatory metabolic pathways, and suboptimal pharmacodynamics (Yao et al., 2021). These challenges highlight the need for novel IDO1-targeting compounds with superior activity, structural diversity, and improved cellular engagement.

To address this unmet need, we have developed SK1281, a rationally engineered tryptophan- and histidine-enriched peptide-like compound with the sequence HFAVKHAVWAHVWHKAKSR (Kakkerla et al., 2025). The design incorporates several features associated with improved binding behavior and cellular penetration, including cationic and amphipathic properties as well as aromatic residues known to interact favorably with the heme-binding region of IDO1 (Li et al., 2025). Such short peptides often exhibit structural flexibility, enhanced membrane association, and target-specific interaction characteristics, suggesting that SK1281 may inhibit IDO1 through direct molecular binding while maintaining favorable biocompatibility.

In this study, we comprehensively evaluate the therapeutic potential of SK1281 as a next-generation IDO1 inhibitor using a multiparametric approach. We first establish its inhibitory effect using recombinant IDO1 enzyme assays. Next, we determine its impact on IFN-γ-induced IDO1 activity in CRC cell lines (HT29 and HCT116), reflecting clinically relevant immunometabolic induction. We further investigate whether IDO1 suppression by SK1281 promotes antitumor activity by assessing cellular viability and apoptosis. Finally, integrating *in silico* docking analysis and structural modeling, we propose a mechanistic basis for SK1281 binding and action. Together, these findings position SK1281 as a promising therapeutic candidate capable of overcoming key obstacles observed with prior IDO1 inhibitors.

2. MATERIALS AND METHODS:

2.1. Reagents and Chemicals

The peptides were synthesized using Fmoc (N-[9-fluorenyl]-methoxycarbonyl) chemistry, as described previously (FIELDS & NOBLE, 1990). In this method, the amino acids were activated in situ with N-hydroxybenzotriazole (HOBt) and N, N-diisopropylethylamine (DIEA). For side-chain deprotection and peptide cleavage from the solid support, a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water was employed. Purification of the peptides was performed using preparative reverse-phase high-performance liquid chromatography (HPLC) with a Kromasil C18 column. Analytical reverse-phase HPLC and electrospray mass spectrometry confirmed the identity and purity of the synthesized peptides. Indoleamine 2,3-Dioxygenase 1 (IDO1) Activity Assay Kit (Sigma – MAK356) was procured commercially. Interferon-gamma (IFN-γ) (Sigma - I3265) was prepared in sterile PBS containing 0.1% BSA according to manufacturer recommendations. All solvents, culture reagents, and analytical-grade chemicals, unless otherwise specified, were obtained from standard suppliers.

2.2. Cell Culture

Human colorectal adenocarcinoma cell lines HT29 and HCT116 were cultured in Dulbecco's Modified Eagle Medium (DMEM; high glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin–streptomycin, and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 and sub-cultured at 70–80% confluence using 0.25% trypsin-EDTA.

2.3. Recombinant IDO1 Enzyme Inhibition Assay

The ability of SK1281 to inhibit IDO1 catalytic activity was evaluated using a fluorometric kynurenine quantification assay. Recombinant human IDO1 enzyme was incubated with assay buffer containing ascorbate, methylene blue, catalase, and L-tryptophan substrate according to the supplier's protocol. SK1281 was tested at 1, 2, and 10 µg/mL, while IDO5L (1 µM) served as the

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positive control. After incubation at 37 °C for 60 min, the conversion of tryptophan to kynurenine was quantified using a fluorescence microplate reader (Ex/Em: 402/488 nm). Results were expressed as % inhibition relative to vehicle-treated enzyme control.

2.4. IFN-γ Induction and Cellular IDO1 Activity Assay

HT29 and HCT116 cells were seeded in 96-well plates (1 × 10^4 cells/well) and allowed to adhere overnight. Cells were stimulated with IFN-γ (100 ng/mL) for 24 h to induce IDO1 expression. Subsequently, SK1281 (1, 2, or 10 µg/mL) or vehicle control was applied for an additional 24 h. Cells and culture supernatants were processed according to the fluorometric IDO1 activity kit instructions. Enzymatic activity was normalized to protein concentration and expressed as pmol/min. All experiments were performed in triplicate.

2.5. Cell Viability Assay

Cell viability following SK1281 exposure was assessed using an MTT assay. HCT116 cells were seeded in 96-well plates (1 × 10^4 cells/well) and treated with SK1281 across a wide concentration range (0.01–1000 µg/mL) for 24 h under standard culture conditions. After incubation, assay reagent was added, and absorbance (570 nm for MTT) was measured on a multimode reader. Cell viability was expressed relative to untreated controls.

2.6. Apoptosis Quantification

Apoptotic response was evaluated using Annexin V-FITC/propidium iodide (PI) dual staining. HT29 and HCT116 cells were seeded in 6-well plates (2 × 10^5 cells/well), induced with IFN-γ (100 ng/mL), and treated with SK1281 for 24 h. Cells were harvested by trypsinization, washed with cold PBS, and stained with Annexin V-FITC and PI according to the manufacturer's protocol. Samples were analyzed via flow cytometry using a 488-nm excitation laser. Data were processed using quadrant gating to differentiate viable, early apoptotic, late apoptotic, and necrotic populations. A minimum of 10,000 events was recorded per sample.

2.7. In Silico Molecular Docking

The amino acid sequence peptide SK1281 was initially submitted to Blastp to search for structural homologs for homology modelling; however, no significant structural homology was identified. Consequently, the sequences were submitted to the I-TASSER server (Roy et al., 2010) for 3D structural prediction. The server generated the top five predicted structures with high C-scores. From these, a structure that was consistent with the Ramachandran plot was selected for further refinement. Energy minimization of the selected structure was performed using the steepest descent algorithm via the NOMAD-REF online program (http://lorentz.dynstr.pasteur.fr/docking/submission.php). The energy-minimized structure was then used for molecular docking simulations on human IDO1(6E45) using the ZDOCK server (http://zdock.umassmed.edu) (Pierce et al., 2014). The docking program generated the top 10-scoring complexes, considering factors such as shape complementarity, electrostatics, and rigid-body interactions.

2.8. Statistical Analysis

Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Data are presented as mean ± standard deviation (SD) from at least three independent biological replicates. A significance threshold of $p < 0.05$ was considered statistically meaningful.

3. RESULTS

3.1. SK1281 Inhibits Recombinant IDO1 Enzyme Activity

The recombinant IDO1 enzyme assay demonstrated that SK1281 is a potent inhibitor of IDO1 catalytic activity. IFN-γ–induced vehicle control groups showed a robust increase in kynurenine fluorescence, confirming active enzyme function. Upon treatment with SK1281, a clear dose-dependent inhibition was observed. At 1 µg/mL, SK1281 significantly reduced the enzymatic conversion of tryptophan, and this inhibition further intensified at 2 µg/mL. The highest evaluated concentration, 10 µg/mL, almost completely blocked enzyme activity and exhibited comparable inhibitory potential to the standard reference inhibitor IDO5L (1 µM). (Figure 1). These results indicate that SK1281 directly interacts with the IDO1 catalytic center, likely preventing substrate access to the heme active site and therefore effectively disrupting tryptophan metabolism at the enzymatic level.

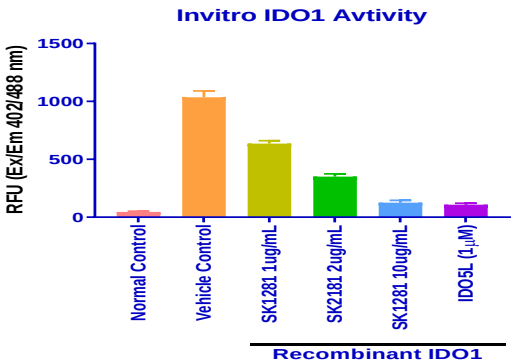

Figure 1: Dose-dependent inhibition of recombinant human IDO1 enzyme by SK1281.

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Recombinant IDO1 was incubated with SK1281 at concentrations of 1, 2, and 10 µg/mL, or with the reference inhibitor IDO5L (1 µM). Kynurenine formation was quantified by fluorometric assay (Ex/Em 402/488 nm). Results are expressed as percentage enzyme activity relative to vehicle control.

3.2. Molecular Docking Results

To rationalize the inhibitory activity of SK1281 against IDO1, in silico molecular docking studies were performed using the crystal structure of human IDO1. The predicted 3D conformation of SK1281 revealed a predominantly α-helical backbone with amphipathic character, enabling optimal alignment within the enzyme's active site. The best-scoring docking pose demonstrated a stable and complementary interaction between SK1281 and the catalytic pocket of IDO1, yielding a strong binding affinity consistent with competitive inhibition (Figure 2). Notably, SK1281 situated its two aromatic tryptophan residues (Trp9 and Trp13) in proximity to the heme prosthetic group, enabling π–π stacking and hydrophobic stabilization that likely prevents substrate access to the catalytic center. Additionally, a key hydrogen bond was observed between His11 of the peptide and Ser167, a residue known to contribute to substrate coordination and catalytic turnover. The peptide's positively charged residues (Lys and Arg) formed electrostatic contacts with His346, a residue involved in heme positioning and oxygen-binding dynamics. Together, these interactions are strongly associated with inhibition of dioxygenase function. The amphipathic nature of SK1281 also facilitated hydrophobic interactions with Phe163, Phe226, Leu234, and Val130, effectively anchoring the peptide at the substrate channel entrance while maintaining orientation suitable for heme interference. The observed binding geometry closely resembles the interaction patterns of reference IDO1 inhibitors, supporting that SK1281 can obstruct L-tryptophan turnover at the initial rate-limiting step of kynurenine biosynthesis. Overall, the docking simulations suggest that SK1281 exerts its inhibitory activity via a combination of hydrogen bonding, aromatic stabilization, and electrostatic heme-proximal interactions, leading to potent and selective catalytic blockade. These structural features align well with experimental results showing strong inhibition of both recombinant and cellular IDO1 function, supporting a direct molecular mechanism of action.

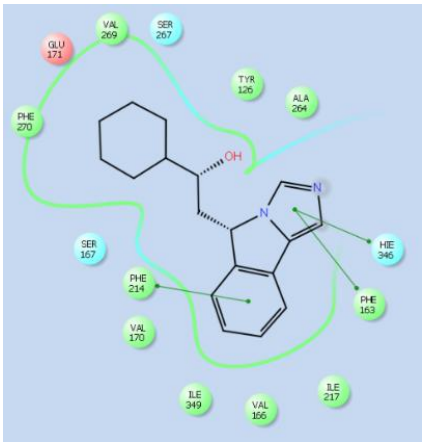

Figure 2. Molecular docking interactions of SK1281 with the IDO1 catalytic site.

3.3. SK1281 Suppresses IFN-γ–Induced IDO1 Activity in Colorectal Cancer Cells

To assess pharmacological effect under biologically relevant conditions, cellular IDO1 activity was measured in IFN-γ–stimulated HT29 and HCT116 colorectal cancer cells. IFN-γ induction significantly elevated IDO1 activity in both lines, representing tumor-associated immune resistance. SK1281 treatment resulted in a marked and dose-dependent reduction in enzyme activity within the cells, demonstrating its ability to penetrate and function in the intracellular environment. At concentrations of 1 and 2 µg/mL, IDO1 suppression was substantial, while 10 µg/mL treatment almost completely neutralized IFN-γ–mediated activation (Figure 3). The consistency of results between the two cancer models suggests that SK1281 is effective across different colorectal cancer phenotypes and capable of reversing tryptophan catabolism-driven immunosuppression.

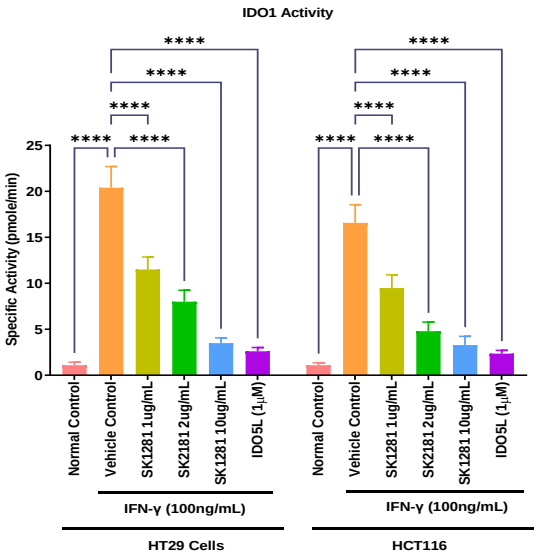

Figure 3. SK1281 suppresses IFN-γ-induced IDO1 activity in colorectal cancer cells.

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HT29 and HCT116 cells were pre-stimulated with IFN-γ (100 ng/mL) for 24 h to induce IDO1 expression and subsequently treated with SK1281 (1, 2, &10 µg/mL) for an additional 24 h. Cellular IDO1 activity was quantified, normalized to protein concentration, and expressed as pmol/min.

3.4. SK1281 Exhibits Minimal Cytotoxicity in Cancer Cells

Cell viability analysis was performed to ensure that SK1281-mediated IDO1 inhibition was not due to nonspecific cytotoxicity. HCT116 cells treated with a wide range of SK1281 concentrations (0.01–1000 µg/mL) retained above 90% viability, with no significant deviation from vehicle controls (Figure 4). These findings confirm that SK1281 does not impair basal cellular integrity or growth in the absence of immunometabolic stress. The favorable safety profile also indicates a selective mechanism of action, as apoptosis-driven effects are attributable to IDO1 pathway inhibition rather than direct chemical toxicity. This selectivity is advantageous for future therapeutic applications where off-target toxicity is a key limitation.

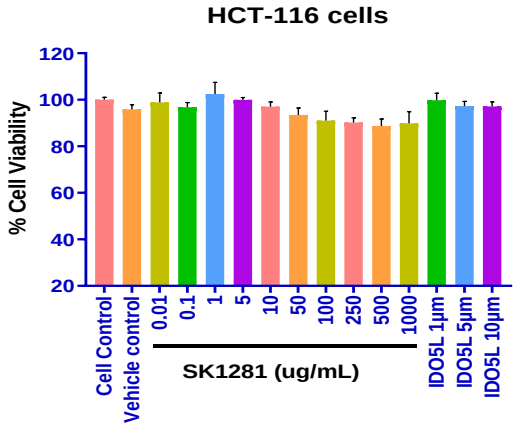

Figure 4: SK1281 exhibits minimal cytotoxicity in HCT116 cells.

Cells were exposed to SK1281 across a wide concentration range (0.01–1000 µg/mL) for 24 h, and viability was assessed by MTT assay. Cell viability is expressed as a percentage of the untreated control

3.5. SK1281 Induces Dose-Dependent Apoptosis in Tumor Cells

Since IDO1-driven kynurenine accumulation promotes tumor cell survival and resistance to immune attack, apoptosis was analyzed to determine the downstream biological effects of IDO1 inhibition. Flow cytometry results revealed that SK1281 significantly increased the apoptotic cell fraction in a dose-dependent manner in both HT29 and HCT116 cells. While untreated and vehicle controls showed minimal apoptotic populations, SK1281 at 1 µg/mL induced noticeable apoptosis, which further escalated at 2 µg/mL. The most dramatic effect was seen at 10 µg/mL, where apoptosis exceeded 50% in both cell lines (Figure 5 A & B). These results suggest that SK1281 successfully overcomes IDO1-mediated anti-apoptotic defenses, restoring programmed cell death mechanisms essential for tumor suppression. Thus, SK1281 not only blocks the immunosuppressive metabolic pathway but also directly triggers antitumor responses.

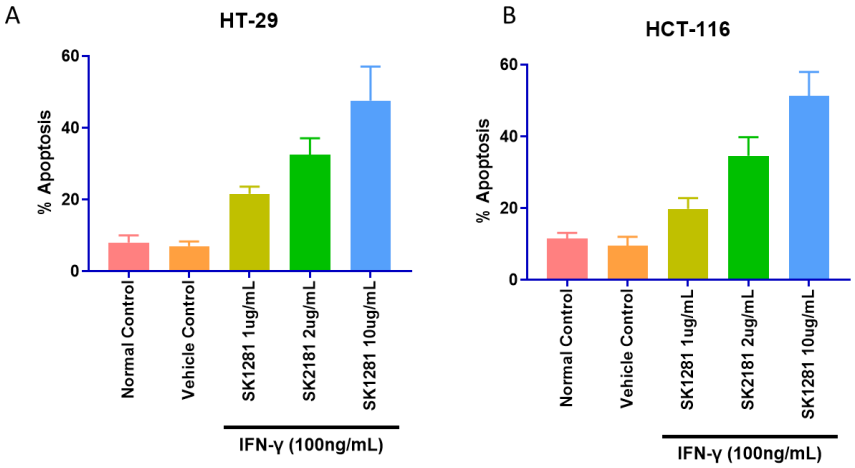

Figure 5. SK1281 induces apoptosis in colorectal cancer cell lines in a dose-dependent manner.

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HT29 and HCT116 cells were treated with SK1281 (1, 2, and 10 μg/mL) following IFN-γ induction, and apoptosis was quantified by Annexin V-FITC/PI dual staining using flow cytometry. Bar graphs represent the percentage of total apoptotic (early + late) cells.

4. DISCUSSION:

Colorectal cancer (CRC) continues to present major clinical challenges due to immune evasion, metastatic progression, and resistance to existing immunotherapies (Ganesh et al., 2019; Sung et al., 2021). In this context, metabolic immune checkpoints such as IDO1 have emerged as critical therapeutic targets because they regulate tryptophan catabolism to kynurenine, which impairs effector T-cell function and supports tumor immune tolerance (Munn & Mellor, 2007b; Platten et al., 2021). In this study, we investigated SK1281, a rationally engineered peptide-based compound designed to exert potent binding to the IDO1 catalytic site while maintaining favorable physicochemical properties enabling cellular entry and target interaction.

Our findings demonstrate that SK1281 effectively inhibits IDO1 activity at both the purified enzyme and cellular levels. The significant and dose-dependent reduction of recombinant IDO1 activity suggests direct catalytic interference, while suppression of IFN-γ–induced IDO1 activity in CRC cells confirms effective intracellular engagement under physiologically relevant immunostimulatory conditions . Importantly, SK1281 exhibited no detectable basal cytotoxicity across a wide concentration range, indicating that enzyme inhibition and downstream cellular consequences are not due to nonspecific damage, but instead result from on-target metabolic modulation. This selectivity is essential for immunotherapeutic drugs, where excessive cytotoxicity may suppress desirable immune responses (Liu et al., 2025).

A central finding of this study is that IDO1 inhibition by SK1281 leads to a marked increase in apoptotic response in both HT29 and HCT116 cells. IDO1 overexpression is known to activate anti-apoptotic survival pathways mediated through kynurenine-AhR signaling (Opitz et al., 2011), and our observation of significant apoptosis following inhibition suggests restoration of pro-death mechanisms normally suppressed by immunometabolic reprogramming. The concentration-dependent nature of this response aligns closely with the graded suppression of IDO1 enzymatic function, reinforcing a mechanistic link between metabolic pathway blockade and antitumor effects.

The molecular docking and structural modeling results provide important mechanistic insight supporting the biochemical observations. SK1281's amphipathic α-helical structure allowed favorable accommodation into the IDO1 catalytic heme pocket. Hydrogen bonding with Ser167, electrostatic interactions with His346, and π–π stacking proximity of tryptophan residues to the heme center collectively form a highly stable binding configuration consistent with that of known IDO1 inhibitors (Günther et al., 2019). Such interactions are associated with competitive substrate displacement and catalytic inactivation.

Collectively, these data support SK1281 as a dual-function therapeutic candidate capable of (i) reversing tumor-driven tryptophan depletion and kynurenine accumulation, and (ii) promoting direct CRC cell apoptosis through immunometabolic reactivation. These properties are highly relevant given the disappointing outcomes of earlier IDO1 inhibitors in clinical trials (Tang et al., 2021), which suffered from inadequate intratumoral efficacy and limited modulation of downstream survival pathways. Peptide-based inhibitors like SK1281 introduce structural diversity and may overcome pharmacokinetic and tumor penetration limitations observed with small molecules.

In conclusion, SK1281 demonstrates strong therapeutic promise as a next-generation IDO1 inhibitor capable of restoring antitumor metabolic balance and inducing apoptosis in colorectal cancer cells. These findings warrant continued investigation in more complex immunocompetent models and evaluation in combination with immune checkpoint blockade to maximize clinical impact.

5. CONCLUSIONS:

In this study, we characterized the biological activity of SK1281, a newly designed amphipathic peptide targeting the metabolic immune checkpoint enzyme IDO1. SK1281 demonstrated potent and dose-dependent inhibition of IDO1 catalytic activity in both recombinant enzyme assays and IFN-γ–induced colorectal cancer cell models, confirming effective intracellular engagement of the tryptophan–kynurenine pathway. Unlike nonspecific cytotoxic agents, SK1281 exhibited a favorable safety profile across a broad concentration range, while significantly inducing apoptosis in colorectal cancer cells in a concentration-dependent manner. These findings collectively indicate that SK1281 restores tumor-suppressive signaling that is otherwise suppressed by IDO1-mediated immunometabolic reprogramming.

Molecular docking provided mechanistic insight supporting experimental results, revealing placement of SK1281 near the heme coordinating region with key hydrogen bonding and hydrophobic interactions consistent with competitive inhibition. Given limitations observed with previous generations of IDO1 inhibitors in clinical trials, SK1281 provides structural innovation and improved functional potency that warrant its continued preclinical development.

Overall, SK1281 represents a promising next-generation IDO1 inhibitor with dual direct anticancer and immunomodulatory potential. Future investigations will focus on defining its effects in co-culture immunocompetent systems, evaluating antitumor efficacy in vivo, and exploring its compatibility with immune checkpoint blockade as part of combination immunotherapy strategies for colorectal cancer.

Data Availability Statement:
All data generated or analysed during this study are included in this published article and its supplementary information files. Further information is available from the corresponding author upon reasonable request.

FUNDING
CDU institutional research fund, SK. fellowship eas supported by CDU institutional fellowship.

ACKNOWLEDGMENTS:
The authors would like to thank the Chancellor, CDU, Dr. Chintalpani V. Purushotham Reddy, and the Vice Chancellor, CDU, for providing the necessary infrastructural facilities and support for the execution of the above study. SK is supported by a CDU institutional fellowship and registered for the Ph.D. program at CDU, Hyderabad.

Author's Contribution:
SK, Sridhar K, and SC conceived the idea and designed the experiments. SK and Sridhar K performed the experiments. SK, Sridhar K, and SC analyzed the data. Sridhar K made all the figures of the manuscript and did the statistical analysis. SK and Sridhar K wrote the initial draft, and SC edited the manuscript. All authors approved the final version of the manuscript.

Conflict of Interest:
The authors declare no competing financial interests and no conflict of interest.

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