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Dual targeting of TGF-β and PD-L1 inhibits tumor growth in TGF-β/PD-L1-driven colorectal carcinoma

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Abstract: Immunosuppressive factors within the tumor microenvironment (TME), for example TGF-β, constitute a crucial hindrance to immunotherapeutic approaches in the management of colorectal cancer (CRC). Furthermore, immune checkpoints (e.g., PD-L1) inhibit T cell proliferation and activation. To cope with the inhibitory effect of immune checkpoints, the therapeutic value of dual targeting of the PD-L1 and TGF-β pathways using M7824 plus 5-FU in CRC has been evaluated. Integrative-systems biology approaches and RNAseq were used to assess the differential level of genes associated in 88 metastatic-CRC patients. The level of PD-L1 and TGF-β was evaluated in a validation cohort. The anti-proliferative, migratory, and apoptotic effects of PD-L1/TGF-β inhibitor, M7824, was assessed by MTT, wound-healing assay, and flow cytometry. Anti-tumor activity was assessed in a xenograft model, followed by biochemical studies and histological staining, and gene/protein expression analyses by RT-PCR and ELISA/IHC. The result of DEGs analysis showed that there were 1268 upregulated and 1074 downregulated genes in CRC patients. PD-L1 and TGF-β were found to be among the top-scoring genes and dysregulated pathways associated with CRC, and this was validated in 92 CRC patients. Targeting of PD-L1-TGF-β inhibited cell growth and migration, associated with modulation of CyclinD1 and MMP9. Furthermore, M7824 inhibited tumor growth via targeting TGF-β and PD-L1 pathways, resulting in modulation of inflammatory response and fibrosis via TNF-α/IL6/CD4-8 and COL1A1/1A2, respectively. In conclusion, our data indicate that co-targeting the PD-L1 and TGF-β pathways increased the effect of 5-FU and reduced the tumor growth in PD-L1/TGF-β expressing tumors, providing a new therapeutic option in the treatment of CRC.

Keywords: Colorectal Cancer; Bifunctional fusion protein; PD-L1/ TGF-β; Targeted therapy

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1. Introduction

Colorectal cancer (CRC) is amongst the most common causes of cancer-related deaths (1). The survival rate can be up to five years and is different for CRC patients, ranging from 90% to 14% for patients diagnosed with localized disease and those diagnosed with advanced stages, respectively (1). Current standard first-line chemotherapeutic regimens are FOLFOX and FOLFIRI (2), but the efficacy of these regimens is limited by systemic toxicity and primary/acquired resistance (3, 4). Targeting immune checkpoint molecules such as PD-1, and PD-L1 has emerged as a promising therapeutic approach in cancer treatment (5, 6). Programmed death-ligand 1 (PD-L1), also termed CD274, is a membrane-bound protein expressed in the lymphocytes, monocytes, natural killer T cells (NK cells), T cells, and B cells. PD-L1 is expressed in the tumor cells and causes immune-suppressive. A high level of the PD-L1 receptor has been found in different malignancies. It interacts with the PD-1 receptor expressed on T- and NK cells. This interaction suppresses the immune system by inhibiting maturation and proliferation. Therefore, the activation of immune cells has been the target of several immune checkpoint inhibitors (ICIs) (7). However, a significant obstacle to the success of tumor immunotherapy is TGF-β, which plays an important role in immune suppression by inhibiting T cells and NK-cells. TGF-β is a cytokine belongs to the transforming growth factor superfamily which secreted by various cell types, including parenchymal, macrophages, monocytes, lymphocytes, and platelets (8-12). Avelumab is a human monoclonal antibody approved by the FDA for treating Merkel cell bladder carcinomas, and urothelial carcinoma (13, 14). M7824, the first class of bifunctional fusion proteins, comprising IgG1 similar to Avelumab, and two extracellular domains of human TGFβRII connected to the C-terminus of the anti-PD-L1 heavy chain of avelumab. The TGFβRII of M7824 inhibits the TGF-β signaling pathway by trapping three forms of TGFβ. Therefore, the novel monoclonal antibody is expected to promote anti-tumor efficacy by blocking both PD-L1 and TGFβ pathways (15). A previous study showed that M7824 was able to revert EMT properties by decreasing the expression of mesenchymal markers, tumor plasticity, and chemo-resistance (16). Another study showed that M7824 enhanced antibody-dependent cell-mediated cytotoxicity by increasing NK cell activity and decreasing the activity of regulatory T cells (17). Furthermore, a clinical study (MSB0011359C) has confirmed the efficiency and safety of M7824 (18). In our current research, we investigated the anti-tumor efficacy of targeting TGF-β + PD-L1 using M7824 alone for the first time and also along with gold-standard chemotherapy in CRC, 5-Fluorouracil (5-FU), in in-vitro and in-vivo models of CRC.

2. Materials and Methods

2.1. Drugs and chemicals

M7824 was purchased from Abcam Company (Abcam, MA, USA). RPMI 1640 medium, Fetal bovine serum, 50 units/mL penicillin, and 50 units/mL streptomycin were purchased by BetaCell, Tehran, Iran.

2.2. Cell culture

SW-480, HT-29, and CT-26 were purchased and certified from the National Cell Bank of Iran (NCBI) based at the Pasteur Institute of Iran (IPI). All the cell lines were cultured in RPMI or DMEM, plus 10% FBS and 1% streptomycin/penicillin. The cells were kept at 37°C in a 5% CO2 atmosphere.

2.3. Patient samples
Eighty-eight CRC tumors and their corresponding normal margins were enrolled based on histological confirmation by two pathologists. All the eligible patients were chemotherapeutically naive patients treated in Omid Hospital of MUMS. Supplementary Table 1 is abridged from Details on diagnosis and treatments. The study was approved by the local Hospital Ethics Committee of MUMS.

2.4. Growth inhibition studies

Cell viability was evaluated using the MTT assay after 72-hour treatment. Cell lines were treated with M7824 (1-100 μg), 5-FU (1 to 500 μM), and the combination of M7824 and 5-fu on IC50 values of each drug. Finally, the absorbance of wells was investigated at 570 nm by using an Eliza reader (Epoch, BioTek, USA) (17).

2.5. Evaluation of pharmacological interaction of M7824 and 5-FU

We evaluated the pharmacological interaction of M7824 with 5-FU by comparing the cell growth of drug alone and in combination, using the combination index (CI), where CI indicated in the synergistic is less than 0.9, additive is between 0.9-1.1, and antagonistic is more than 1.1. Simultaneous treatment of M7824 fixed concentration and six different concentrations of 5-FU was performed to investigate the nitratin between treatments. CI -fraction affected (FA) values were average of 0.5, 0.75, and 0.9 CI for each group. The mean between different groups was calculated using CalcuSyn software version 2.0 (Biosoft, Oxford, UK).

2.6. ELISA

Cells (1.0*10^6) were seeded into the 6-well plate and grown-up for 72 h in RPMI, plus 10% FBS and 1% streptomycin/penicillin. The expression levels of TGF-β (Bioassay Technology Laboratory, Korea), IL-6, and TNF-α (IBL Intentional GMBH, Hamburg, Germany) were evaluated after therapy in CRC cells as well as cytokines secreted by the cells used culture media supplemented with 10% FBS as control group.

2.7. Multicellular spheroids

Initially, 1g agarose was dissolved in 100 ml RPMI (1% agarose), heated to 90 degrees for a few minutes, 10 μl loaded at the bottom of wells, and allowed to solidify completely. Then, the 105 cells/mL in a complete cell culture medium in agarose-coated 96-well plates were seeded to form multicellular spheroids. The cells were followed (for four days) using an inverted phase-contrast microscope. ImageJ measured the spheroid diameter, and spheroid volume was calculated as described previously (18).

2.8. qRT-PCR

RNA was isolated (Parstous, Tehran, Iran) and were assessed by a Nanodrop 2000 spectrophotometer (BioTek, USA EPOCH). cDNA was synthesized according to the manufacturer’s instructions (Parstous, Tehran, Iran). Quantitative real-time PCR was performed using specific primers, as shown in the supplementary table 2 (Macrogene Co., Seoul, South Korea) and SYBR green master mix (Parstous Co. Tehran, Iran) using an ABI-PRISM StepOne™ instrument (Foster City, CA) (18). To identify tissue-specific housekeeping genes for gene expression analysis and to avoid single control normalization error, accurate normalization of
qRT-PCR data based on the geometric means of multiple internal control genes was performed. The housekeeping gene which was used as internal control was GAPDH. 2.9. Quantification of apoptosis

AnnexinV/P.I. Apoptosis Detection Kit (MabTag, GmbH, Germany) was used to determine cellular apoptosis. Cells were treated with M7824, 5-FU, at IC50 for 72 hours. The proportion of apoptotic cells was investigated by BD FACSCalibur (BD Biosciences, San Jose, CA) (19).

2.10. Animal model

Pasteur Institute (Tehran, Iran) supplied female mice of 8-week-old inbred BALB/c. The animal experiments were ethically approved under regulations set by the Animal Care Committee of Mashhad University of Medical Science guidelines. CT-26 is a murine colorectal carcinoma derived from BALB/c mice, and the syngeneic tumor model is one of the most commonly used to create the CDX (Cell Line Derived Xenograft) CT26 syngeneic murine model. To establish subcutaneous tumors, animals were inoculated with a suspension of CT-26 cells (2x10^6 per mouse) injected into the animal’s right flank region. After two weeks, the tumor-bearing mice with a tumor volume that reached 80–100mm^3 were randomly divided into three groups (n = 4): Control, treated with m7824 at a dose of 50μg, and 5-FU. Tumors were calculated three times during each week using calipers, and the tumor volume was calculated as described (18).

2.11. Histopathological staining

Tumor tissues were stained with hematoxylin and eosin (H&E), trichrome, and then were examined by at least 2 investigators using light microscopy (×40 magnification).

2.12. Immunohistochemical staining

The level of CD4+/CD8+ was evaluated by immunohistochemical staining using specific antibodies against CD4+/CD8+, as described previously (17).

2.13. In silico analysis

The orientation of M7824 in the target proteins’ active site was analyzed using MOE (http://www.chemcomp.com). It is the first time that the association of the interaction and inhibitory effects of the mentioned proteins in this study by M7824 has been investigated. In this study, we drew the structure of M7824 using a chemical structure drawing program (ChemDraw Ultra 7.0) and minimized its energy by MOE. The crystal structure of proteins were downloaded from the RCSB Protein Data Bank. PDB IDs of the study were 3TZM (for TGF-β1), 2M2D (for PD-1), 1KS6 (for TGF-β2), 1lb5 (for TRAF6), 1YG5 (for SMAD4), 6M64 (for SMAD2), 1mk2 (for SMAD3). The concluding scores were assessed using the GBVI/WSA dG scoring (20, 21) and estimates the binding free energy of the ligand from a presented pose. Ki (inhibition constant) was calculated by the binding free energy estimated with the GBVI/WSA dG scoring function [ΔG=RTln(Ki)]. In this equation, T represents the temperature in kelvin and R the gas constant. The pKi was calculated from the binding free energy values at 298 K (a fixed temperature) by using the equation [log Ki = pKi].
2.14. Identifying dysregulation expression genes (DEGs)

Gene expression profiling was performed in 9 CRC cases (ID: SRA study: SRP029880). FeatureCounts package was implemented in Linux (ubuntu 20.4 LTS) to generate a matrix of gene-wise counts as input for expression analysis package. All the data were normalized, and then DEGs were analyzed based on R software (v.3.6), using the package of DESeq2 (v.1.20). Previously the official gene symbols corresponding to the Ensembl gene ID were annotated and then conformed to the human GFF3 specifications using the biomaRt Bioconductor package. Then, we contrasted two conditions, CRC patients with advanced-stage versus normal cases. They were considered as input for obtaining up and downregulated genes by DESeq2 and Clusterprofiler package to analyze Gene Set Enrichment and apply AnnotationHub package for revealing Disease Ontology, Network of Cancer Gene, Gene Ontology, and KEGG Pathways. Meanwhile, gene ontology, including biological process, molecular function, and cellular component, was designed by Enrichr to determine the mutual roles of accumulative genes being interacted in the cell. For the current research, special criteria were performed, including P-values < 0.05, and Log2-fold change |1| >. Statistical significance was considered at p < 0.05, *p < 0.05, **p < 0.005, ***p < 0.001.

2.15. Statistical analysis

Statistical analyses were analyzed by SPSS v20 software with one way anova test and it’s post test (Tukey-Kramer tests), and deviation t-test statistical significances were inferred at p ≤ 0.05 using Graph-Pad Software (version 8). The experiments were undertaken in duplicate and verified in at least two independent experiments.

3. Results

3.1. Gene signatures and PD-L1/TGF-β expression in CRC patients

The demographic and clinicopathological data of patients are listed in the supplementary Table 1. The result of the DEGs analysis revealed that 1268 and 1074 genes were upregulated and downregulated. Heatmap of the clustering of genes versus patients filtered based on Log2FoldChange > ±1 and p.val < 0.05 was generated (Fig.1A).
Figure 1. Gene signatures and PD-L1/TGF-β expression in CRC patients. A) Hierarchically clustered genes. B) Molecular function, biological process, and cellular component. C) The top 40 ranked pathways simultaneously activated or suppressed by enriched up and down-regulated genes in the samples. D) The gene concept complexities in which the number of involved genes contains crucial biological terms were displayed in colorectal cancer vs Normal samples in a cnetplot. Moreover, the nodes labeled by the selected subset were also depicted in separate groups. E) PPI network constructed with the differentially expressed genes. Red to yellow nodes represent hub GENE analysis by cytoHubba representing high and low rank, respectively. F) The level of PD-L1 and TGF-β in tumor tissue, as de-
The plots generated by autodock and LigPlot+ software show interaction between M7824 and PD-L1 and TGF-β. Hydrogen binding pockets of PD-L1/TGF-β and M7824 (green line) and Hydrophobic interactions (red line). The result showed a dramatic difference in gene expression between the two groups of samples. The top 40 ranked pathways that were activated or suppressed by the up or down-regulation of genes in the pathways are shown in Fig.1B, C. The data also show the most related pathways associated with CRC with a high score of their association with CRC (fig.1D, E). Among the top-scoring genes in the list were PD-L1 and TGF-β and were associated with the poor prognosis. To further validate this data, we evaluated the level of PD-L1 and TGF-β in CRC patients. Mean expression of PD-L1 (Mean±SD=6.4±1.3) and TGF-β (Mean±SD=3.76±5.9) were higher in tumor cells (P<0.05) (Fig.1F). The data illustrated a positive correlation between PD-L1 and TGF-β (P=0.001). Figure 1G and H demonstrated the interaction of M7824 with the targets, PDL-1, and TGF-β.

3.2. M7824 inhibits cell growth

An increasing dose of M7824(1-40 μg/ml) and 5-FU (10-100 μM) was used to treat the HT-29, SW-480, and CT-26 cell lines for 72 hours to investigate its anti-proliferative effects. We observed that different doses of M7824 could stop cell growth in various levels, with an IC50 of 20 μg/ml (fig.2A-C). The combination of M7824 with 5-FU demonstrated that 5-FU synergistically reduced the dosage of M7824 in HT-29 and CT-26 cells. Then the expression levels of TGF-β were assessed. The results demonstrated that treatment of SW-480 and HT-29 cells with M7824 significantly decreased the mRNA levels of TGF-β and PD-L1 (fig.2D). In addition, the level of TGF-β in CRC cells was reduced (Fig.2E). To assess the effect of M7824 on cell cycle, the expression of cyclin D1 and survivin were analyzed. Our data revealed the downregulation of cyclin D1 in HT-29 and CT-26 cells, while there was no difference between in SW-480 (Figure 2F).

The further evaluate the co-targeting of PD-L1 and TGF-β in CRC spheroid overcoming the major obstacle of conventional two-dimensional cell cultures, the spheroids were generated after 24 hr for the SW-480 cell line as well as 48 hr for HT-29 and CT-26 cell lines, and then treated with M7824, 5-FU, and combination at IC50 values in SW-480, HT-29, and CT-26. M7824 was able to decrease the volume of CRC spheroids compared with untreated groups (Fig. 2G, H).

3.3. Co-targeting of PD-L1/TGF-β decreases migration and increases cell death

We also assess the co-targeting of PD-L1/TGF-β on cell migration and cell death in SW-480, HT-29, and CT-26 (Figure 3C). M7824, plus 5-FU, increased cell apoptosis, as shown in figure 2G. The Annexin/P.I. assay revealed a significant induction of apoptosis in the cells treated with M7824 via modulation of Bcl2 (Fig.3A, B, C).

Moreover, although our data showed that M7824 inhibited the cell migration of HT-29 and CT-26 after 48 hours, this effect in SW-480 cells was detected after 72 hours via perturbation of matrix metalloprotease-9 (MMP9), and VEGFR (Fig.3 D-G).
Figure 3. Co-targeting of PD-L1/TGF-β reduces the migration of tumor cells and increases cell death. A, B) Effects of M7824, 5-FU on cell death after 72hr as detected by AnnexinV/P.I. staining. The proportion of apoptotic cells was detected by BD FACSCalibur. C) Modulation of P53 and Bcl2 at mRNA levels after 72 hours of exposure to M7824. D) Results of the wound-healing assay in SW-480, HT-29, and CT-26 cells (insert: representative picture at 24, 48, 72 hours). Cells were exposed to M7824 at 5xIC50 values. E-G) Modulation of MMP9 and VEGFR1 mRNA levels in CRC cells after 72 hours of exposure to M7824. *Significantly different from controls

3.4. inhibition of the pathways suppress tumor growth and fibrosis

To further explore the targeting of PD-L and TGF-β pathways in CRC, we treated the mice with 5-FU and M7824 at IC50 values. As indicated in Figure 4A,B, dual inhibition of
PD-L and TGF-β pathways via M7824 significantly decreased tumor size (P<0.05). To determine the therapeutic value of M7824 on fibrosis, we stained the tissue using Masson’s trichrome stain. The results revealed that M7824 reduced fibrosis and collagen content in tumor tissue via reduction of ColA1A (Fig. 4C-F).

**Figure 4.** M7824 inhibits growth and fibrosis. A) Experimental design of the study in the in vivo setting; B) tumor growth and weight; C) H&E of tumor tissues (×10). Tumor tissue exhibited aggregation of tumor cells (T) and necrotic area (N); D) trichrome staining (×10).
Tumor cell, N; Necrosis tissue, Arrow; Fibrosis tissue. (E,F) M7824 significantly decreased collagen content and Col1A1 in treated mice and cells. Suppression of inflammatory markers and activation of the immune system. Effect of M7824, 5-FU, and their combination treatment on the concentrations of (G) TNF-α, and (H) IL-6 on three cell lines, SW-480, HT-29, and CT-26 at protein level, evaluated by ELISA. (I) IHC staining of tumor tissues by CD4 and CD8 antibody. M7824 promotes the activation and proliferation of T-cells.

3.5. Activation of the immune system

Several studies have reported the cross-regulation of PD-L1 and TGF-β pathways and their roles in suppressing the immune system. In turn, it has been illustrated that dual targeting of these pathways could synergistically enhance the anti-tumor activity of Oxa/5-FU and radiotherapy, due to activation of CD8+ T cells, reducing the EMT (20-21,38) and reducing the level of inflammatory markers such as TNF-α. To examine the potential value of M7824 on T-cells activation and inflammatory response, the expression levels of CD4+/CD8+ and IL-6/TNF-α were evaluated by immunohistochemical staining and ELISA. This data revealed the downregulation of TNF-α and IL6 as well as the increased levels of CD4+ and CD8+ T-Cell in the M7824 treatment group versus control ones (Fig. 4G, H, I).
Figure 5. Docking analysis shows the affinity of M7824 with target proteins TGF-β1, 2, PD-L1, TRAF6, and SMAD2. A) Docking outcomes are confirmed that ligand could directly interact and inhibit TGF-β1 and PD-L1. B) Schematic illustrates the mechanism of action M7824 in the promotion of immune cells by blocking of PD-L1 immune checkpoint and TGF-β1 to decrease EMT and tissue remodeling processes, inflammation, angiogenesis, and fibrosis and increase apoptosis in tumor cells as well.

We performed an in-silico analysis and evaluated the affinity of M7824 (ligand) with target proteins TGF-β1, 2, PD-L1, TRAF6, and SMAD2, 3, 4 (Figure 5A, Supplementary Table 3). Docking outcomes shows that ligand could directly interact and inhibit TGF-β1 and PD-1 according to Fig. 1G,H (Score = -7 and -6 kcal/mol, respectively). Among them, TGF-β1 had pKi = 5.14 through three H-acceptor bonding among oxygen of ligand to LYS_232 residue nitrogen (distance: 3.58) and LYS_335 residue carbon (distance: 3.42), and between ligand
nitrogen with HIS_283 residue nitrogen (distance: 3.53) and one H-pi binding between its 6-ring to Leu_340 carbon (distance: 3.79), followed by PD-1 (pKi = 4.35; one H-acceptor binding of ligand carbonyl group oxygen to VAL_97 residue nitrogen; TGF-β2 (pKi = 4.25; two H-donor binding between ligand hydroxyl group oxygen of LYS_81 and ASP_84 residues with distances 3.31 and 2.83, respectively and one H-donor binding between amide group nitrogen to ASP_83 residue oxygen (distances: 3.16) and one pi-H binding between its 6-ring to GLY_85 residue nitrogen (distance 3.82); TRAF6 (pKi = 4.00; two H-acceptor binding of ligand hydroxyl oxygen to THR_501 residue nitrogen and GLY_386 residue carbon with distances 3.13 and 3.46, respectively and three H-donor binding between nitrogen of amide group, carbon and hydroxyl group oxygen to PRO_385 residue oxygen, in aggregate PD-1 with pKi ≈ 4.3 and TGF-β1 with pKi≈ 5.1, had the highest range of interaction compared with other receptors (Figure 5A).

4. Discussion

Our results demonstrate that M7824 inhibited TGF-β and PD-L1, cell proliferation and fibrosis (Figure 5B). Additionally, M7824 synergistically enhanced the anti-proliferative effect of 5-FU. M7824, a novel class of bifunctional fusion antibodies, targets two different pathways in the tumor microenvironment. The simultaneous functions of M7824 enhance T-cell activity and inhibit the TGFβ pathway. During tumor progression, TGFβ is a critical cytokine in proliferation, migration, metastasis, angiogenesis, and immune evasion (22, 23). The current investigation indicated that TGFβ and PD-L1 expression levels were remarkably increased in CRC patients. Interestingly, a positive relationship was found between TGFβ and PD-L1 levels (p=0.017). And also, our data revealed that M7824 significantly suppressed the mRNA level of TGFβ and PD-L1 in CRC cells.

TGFβ canonical signaling induces phosphorylation of Smad2/3 proteins and forms a complex with smad4, which then binds to Smad-binding elements (SBEs) on targeted genes. The promoter of PD-L1 contains 7 SBEs that can be activated by TGFβ (24). Previous studies are in line with our results; David et al. revealed TGFβ1 promotes the expression of PD-L1 and PD-L2 in NSCLC cells.

Additionally, they showed that blocking Smad2 or Smad3 using siRNA decreased the level of PD-L1 in A549 cells (16). Furthermore, our data illustrated that M7824, 5-FU, and their combination remarkably decreased TGF-β level in the treated cells. Knudson et al. showed M7824 reduced plasma levels of TGF-β in breast and colon mice models. They also showed that a decreasing level of TGF-β modulates the pathway in TME by reducing the phosphorylation of SMAD2 (25).

There is accumulating data that show a high level of PD-L1 in CRC, renal cell carcinoma, osteosarcoma, and breast cancer is related with a poor prognosis. This study provides proof of the concept of targeting PD-L1 or PD-1. Several studies have shown that blocking this pathway could improve patient survival (26-29). PD-1 is a specific receptor on T-cells that interacts with PD-L1 on the tumor cells, repressing T-cells’ activation and proliferation (30). Therefore, both TGF-β and PD-L1 are vital factors in immunosuppression. To evaluate the activity of M7824 on immune system, the CD4/CD8 marker of T-cells was stained by IHC. The results confirmed that the T-cell density was remarkably increased in tumors treated with M7824 compared to untreated mice. Numerous studies reported the effect of M7824 on the activation immune system.

A study on mice bearing EMT6 tumor indicated the frequency of CD8+ T cells and NK Cells increased to about 99% and 75-90%, respectively, after treatment with M7824 (25). In agreement, the treatment of Merkel cell and bladder carcinoma with M7824 showed that the antibody alleviates immunosuppressive activity by regulation of CD4+ T-cells proliferation.
and promotion NK. Cells activity (17). There is also evidence that the immunomodulatory effect of M7824 in urothelial carcinoma cells. M7824 upregulates the expression of CXCL11, a major chemokine contributing to T-cells homing, and also enhances TRAIL-mediated lysis (31). Elevated levels of IL-6 and TNF-α were detected in tissue and plasma of CRC patients, which were correlated with a poor prognosis, metastasis, invasion, and tumor recurrence(32-34). we assessed the levels of IL-6 and TNF-α in treated cells. M7824 reduced the levels of these cytokines by blocking the TGF-β pathway in all three cell lines.

Our data showed that treatment with M7824 caused a differential expression pattern in the MMP9 gene in both SW-480 and CT-26 cells. TGF-β can simulate several MMPs expression in tumor cells. the results revealed that M7824 downregulated several genes such as MMP2, MMP3, MMP9, VIM, ZEB1, and FN1 in lung cancer cells (16). The data revealed that the level of VEGFR gene decreased in the mRNA of level in CT-26 cells, while other cells showed no correlation. A large body of evidence illustrates that activation of VEGFR is a crucial key in tumorigenesis and metastasis. In line with these results, Mazeda et al. showed that high expression of VEGFR in the primary tumor of CRC is related with poor prognosis and survival(35).

The level of cyclin D1 and survivin were investigated, and we found a reduction in the level of cyclinD1, while the treatment did not affect the survivin expression, which is in line with previous report in which TGF-b signaling induces cell proliferation by increasing the leve of cyclinD1, a vital key for the transition cell cycle from G1 to S phase(36). Additionally, TGF-β signaling regulates Wnt canonical signaling through interaction with SMAD3 with β-catenin, preventing β-catenin degradation. One of the critical targets of β-catenin is the cyclin D1 gene which is announced like a novel marker of poor prognosis for CRC patients(37, 38). The further assessments confirmed the anti-proliferative activity of M7824 in three-dimensional cell culture. Knudson et al. showed a strong anti-tumor efficacy for M7824 on breast and colon carcinoma (25). another study found that M7824 decreased tumor volume and the incidence and number of lung metastases. Additionally, the drug markedly extended survival in the treated group relative to the control group(39).

The CRC cells were treated with M7824, and the percentages of apoptotic cells were evaluated by AnnexinV/P.I. assay. A total of ~48 % and 22% of death were observed after M7824 treatment in SW-480 and CT-26 cells, respectively. To further confirm our assumption that M7824 increases cellular death, the expressions of P53, Bcl2, and Bax genes were measured by RT-PCR. Our findings showed Bcl2 expression levels were diminished in treated cells. The apoptotic effect of M7824 is supported by preclinical evidence that revealed the role of M7824 in increasing cell death by the induction of TRAIL in HTB-4 urothelial cancer cells. They showed that M7824 increased cell sensitivity to TRAIL, mediated by TGFβR2(31).

Our data also revealed that M7824 synergistically improved the activity of 5-FU. In line with these findings, previous research revealed that the combination of chemo agents, including M7824 and Oxa/5-FU, with radiotherapy, synergistically enhanced the efficacy of current therapy due to the activation of CD8+ T-cell and reducing the EMT (39).

5. Conclusion

This study shows that co-targeting of TGF-β and PD-L1 pathways enhanced the anti-tumor activity of 5-FU and suppressed tumor growth of CRC cells as a novel approach to CRC treatment. Further studies using CRC spheroids generated in special medium like Dulbecco’s Modified Eagles’ Media:F12 p GlutaMAX-I with insulin-transferrin-selenium, in ultra-low attachment plates and orthotopic mouse models are needed for further evaluation of this new therapeutic approach in the treatment of CRC.
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Authors’ contribution: GK, HF, FR, FA, NS performed the experiments, analyzed the data. GK and NK. prepared the first draft. MH, MK, MG, MM, SS, KA, GF, EG, AA supervised and designed the project. All the authors approved the final version of the manuscript.

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References


8. Travis MA, Sheppard DJAroi. TGF-β activation and function in immunity. 2014;32:51-82.


34. Li T-F, Chen D, Wu Q, Chen M, Sheu T-j, Schwarz EM et al. Transforming growth factor-β stimulates cyclin D1 expression through activation of β-catenin signaling in chondrocytes. 2006;281(30):21296-304.
