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Antagonist of growth hormone-releasing hormone MIA-690 attenuates the progression and inhibits growth of colorectal cancer in mice

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ABSTRACT

Colorectal cancer (CRC) is an aggressive tumor in which new treatment options deliver negative results on cure rates and long-term survival. The anticancer effects of growth hormone-releasing hormone (GHRH) antagonists have been reported in various experimental tumors, but their activity in CRC is unknown. In the present study, we demonstrated that chronic treatment with GHRH antagonist of MIAMI class, MIA-690, promoted survival and gradually blunted tumor progression in experimentally induced colitis-associated cancer in mice, paralleled by reduced inflammation in colon tissue. In particular, MIA-690 improved disease activity index score, and reduced loss of weight and mortality, by improving the survival rates, compared with vehicle-treated group. MIA-690 was also found to reduce various inflammatory and oxidative markers, such as serotonin, prostaglandin (PG)E_2 and 8-iso-$PGF_{2\alpha}$ levels, as well as COX-2, iNOS, TNF-α, IL-6 and NF-kB gene expression. Moreover, MIA-690 inhibited the protein expression of c-Myc, P-AKT and Bcl-2 and upregulated p53 protein expression. In conclusion, we showed that MIA-690 suppresses CRC progression and growth by reducing inflammatory and oxidative markers and modulating apoptotic and oncogenic pathways. Further investigations are required for translating these findings into the clinics.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in adults and it accounts approximately of 10% of cancer-related mortalities in Western countries [1]. Despite early screening and treatment, this tumor represents the fourth leading cause of cancer-related deaths [2]. It is well known that CRC can develop spontaneously or as a late complication of chronic inflammatory bowel disease (IBD), which is characterized by marked inflammation and abnormal activation of various immune cells [3]. In particular, the activation of nuclear factor-kappa B (NF-κB) and various pro-inflammatory cytokines contribute to CRC development and progression, as well as to resistance to chemo- and radiotherapies [4].

Growth hormone (GH)-releasing hormone (GHRH), a hypothalamic neuropeptide that regulates the synthesis and release of GH by the anterior pituitary, is able to exert stimulatory effects in various

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extrapituitary tissues, by binding to pituitary type GHRH receptor (pGHRH-R) [5]. It has been demonstrated that the GHRH antagonists of MIAMI (MIA) class, including MIA-690, show a higher binding affinity for GHRH-R and low endocrine effect on the GH/insulin-like growth factor I (IGF-I) axis [6]. Moreover, MIA-690 displays strong anti-nflammatory and antioxidant effects in different tissues [7,8]. The involvement of P-AKT-STAT-3/NF-kB signalling in GHRH receptor signalling was reported in more than 3 our papers [9–11]. MIA-602, another GHRH antagonist, inhibits the growth, progression and inflammation of human cancer by blocking the P-AKT-STAT-3/NF-kB axis signalling.

However, the potential antitumor role of MIA-690 in CRC remains to be determined. Treatment of animals with a combination of azoxymethane (AOM), a colonic genotoxic carcinogen, and dextran sulfate sodium (DSS), an inducer of colitis, is a well-established model of experimentally induced CRC [12].

In this context, we investigated the long-term effects of MIA-690 on inflammation-associated colon carcinogenesis after treatment with AOM/DSS for 14 weeks, in mice.

2. Materials and methods

2.1. Animals

Adult C57/BL6 male mice (5 weeks old, weight 20–22 g, n = 72) were housed in plexiglas cages (2–4 animals per cage; 55 × 33 × 19 cm), as previously reported [8]. Housing conditions and experimental procedures were strictly in agreement with the European Community ethical regulations (EU Directive n. 26/2014) on the care of animals for scientific research and approved by the Italian Health Ministry (Project n. 885/2018-PR).

2.2. AOM/DSS model of colitis-associated cancer

After 1-week acclimation, mice were randomized into a control group (untreated with AOM/DSS, n = 16), AOM/DSS + vehicle group (n = 28) and AOM/DSS + MIA-690 group (n = 28). In the AOM/DSS-treated groups, mice were injected intraperitoneally with AOM (10 mg/kg body weight; Sigma, St Louis, MO). One week later, 2.5% (w/v) DSS (molecular weight 40 kDa; Sigma Life Science) was added to drinking water, ad libitum for 7 days, followed by 14 days of regular water. Three cycles of DSS treatment were repeated [12,13]. MIA-690 (5 μg) or vehicle solution [0.1% DMSO (Sigma) and 10% propylene glycol] were administered subcutaneously (s.c.) every 24 h for 14 weeks. The injection volume was 0.1 ml for s.c. injection [7,8].

2.3. Clinical disease score

Colitis Disease Activity Index (DAI) scoring was calculated as previously described [13]. DAI score is the combined score of weight loss (0, none; 1, 0–5%; 2, 5–10%; 3, 10–20%; and 4, > 20%), stool consistency change (0, none; 2, loose stool; and 4, diarrhea), and bleeding (0, none; 1, trace; 2, mild hemoccult; 3, obvious hemoccult; and 4, gross bleeding) divided by three. The minimal score is 0 and the maximal score is 4. Animals were treated with anesthetic and analgesic drugs [Caprofen 10 mg/kg; Meloxicam 10 mg/kg; Lidocaine (1–2%) 2 − 4 mg/kg] when they displayed signs of distress, according to the guidelines suggested by the 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3RS).

2.4. Macroscopic and histological examination

After 2 (W2), 5 (W5), 8 (W8) and 14 (W14) weeks, animals (n = 4–6 for each group) were sacrificed by CO_2 inhalation (100% CO_2 at a flow rate of 20% of the chamber volume per min). The colon segment length (from ileocecal junction to the anal verge; mm) and the number of total tumors and their distribution (distal-middle-proximal tract) were recorded [14].

Hematoxylin-eosin analysis in mouse colon was performed as previously reported [8]. Colonic tumors were diagnosed according to description by Ward and our previous studies [13,15].

2.5. Serotonin (5-hydroxytryptamine, 5-HT), prostaglandin (PG)E_2 and 8-iso-$PGF_{2\alpha}$ determination in colon specimens

Levels of 5-HT (μg/mg wet tissue), PGE_2 and 8-iso-$PGF_{2\alpha}$ (pg/mg wet tissue) in colon specimens were evaluated at 2 weeks through high-performance liquid chromatography (HPLC) apparatus and radioimmunoassay (RIA), respectively [14]. Colon specimens dissected from C57BL/6 (n = 4) mice untreated with AOM/DSS were used as positive control.

2.6. RNA extraction, reverse transcription and real-time reverse transcription polymerase chain reaction (RT-PCR)

Gene expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, interleukin (IL)− 6, and NF-kB in colon specimens was determined at 5 weeks by quantitative real-time PCR, as previously reported [16,17].

2.7. Western blot analysis

Western blotting in colon tissues was performed as previously described [18]. Colon samples were homogenized by Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) in RIPA buffer added with 1 mM PMSF (Phenylmethanesulfonyl Fluoride) and protease and phosphatase inhibitors cocktails (Sigma, St. Louis, MO, USA). Homogenized samples were sonicated and centrifuged at 15,000 rpm (4 °C for 20 min). Protein lysate quantification and immunoblot analyses were performed as previously reported [18]. The membranes were blocked in 5% nonfat dry milk and incubated overnight at 4 °C with the appropriate primary antibodies. Rabbit polyclonal AKT, rabbit polyclonal phospho-AKT (Ser473) and rabbit monoclonal p44/42 MAPK (Erk1/2) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse monoclonal c-Myc (9E10), p53 (A-1), and Bcl-2 (C-2) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) were used as appropriate. The blots were revealed by the Westar ηC Ultra 2.0 chemiluminescence substrate (Cyanagen, Bologna, Italy) [18]. GAPDH and β-actin were used as loading control (Santa Cruz Biotechnology, Dallas, TX, USA).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). All data were collected from each of the animals used in the experimental procedure and means ± SEM were determined for each experimental group and analyzed by unpaired *t*-test (two-tailed P value) and 2-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. F values are referring to repeated measure 2-way ANOVA. Statistical significance was accepted at $P < 0.05$. As regards the gene expression analysis, the comparative $2^{-\Delta\Delta Ct}$ method was used to quantify the relative abundance of mRNA and to determine the relative changes in individual gene expression (relative quantification) [17].

3. Results

3.1. GHRH antagonist MIA-690 inhibits colitis and the progression of colitis-associated CRC in an AOM/DSS model

The colitis-associated CRC was induced by a single AOM injection, followed by three cycles of 2.5% DSS oral administration, as in Fig. 1, panel A (study protocol). During fourteen weeks of observation, all AOM/DSS treated animals (AOM/DSS + vehicle and AOM/DSS + MIA-690) showed higher DAI score (Fig. 1, panel B), significant weight loss (Fig. 1, panel C) and higher mortality (Fig. 1, panel D), compared to control group (untreated with AOM/DSS). In this context, the survival rates became increasingly rare as the weeks passed (Fig. 1, panel E). The highest mortality was observed at 8 and 14 weeks in both AOM/DSS +

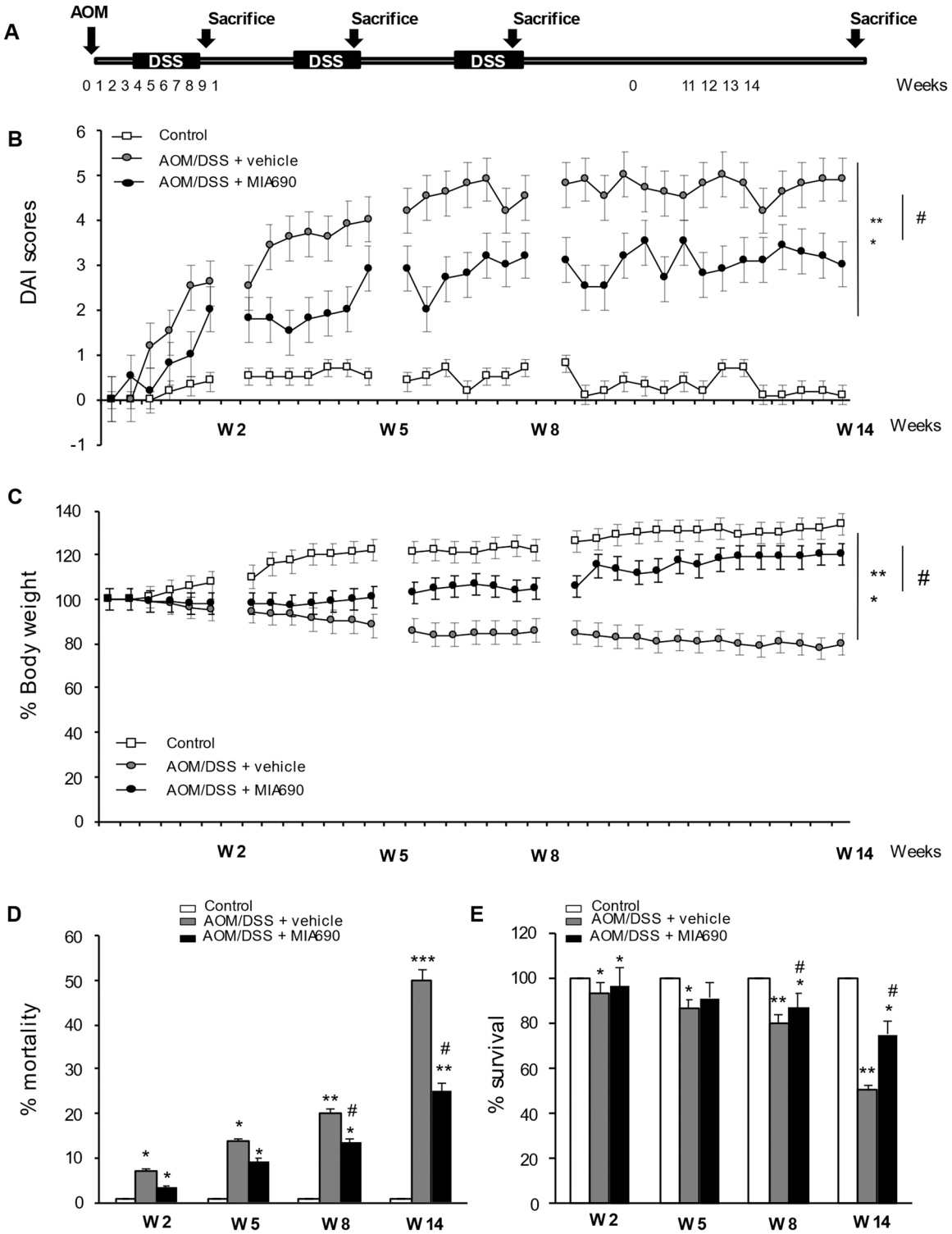

Fig. 1. Effects of s.c. administration of MIA-690 (5 μg) in the AOM/DSS mouse model. Experimental design (A), relative DAI scores (B), percentage (%) body weight (C), mortality (D) and survival (E) in AOM/DSS-treated mice. Data are expressed as means ± S.E.M. (n = 16–28 for each group); * $p < 0.05$, * * $p < 0.005$ and * ** $p < 0.001$ vs. control mice (untreated with AOM/DSS); $^{\#}p < 0.05$ vs. AOM/DSS + vehicle group.

vehicle and AOM/DSS + MIA-690 groups. However, chronic treatment with the GHRH antagonist, MIA-690 (5 μg), improved DAI score and had a positive impact on body weight from the second week onwards (Fig. 1 panel B and C). The AOM/DSS + MIA-690 group also showed a significant reduction in mortality and increased survival rates from the fifth week (W5 = 90,90%, W8 =86,66% and W14 =75%) compared with vehicle group (W5 =86,36%, W8 =80% and W14 =50%) (Fig. 1, panel D and E). Overall, mice treated with MIA-690 showed reduced toxicity after treatment with AOM/DSS treatment.

In order to investigate the potential beneficial effects induced by MIA-690, a limited number of animals (but sufficient for statistical analysis) was sacrificed at 2, 5, 8 and 14 weeks from each group of treatment. After accurate macroscopic evaluation, we observed reduction of colon length and marked inflammation of tissue in all AOM/DSS-treated animals (AOM/DSS + vehicle and AOM/DSS + MIA-690) compared to controls (untreated with AOM/DSS) (Fig. 2, panel A), at 2 weeks. However, AOM/DSS-induced reduction of colon length and inflammation was decreased by MIA-690 (5 μg) treatment as compared to vehicle injected animals (Fig. 2, panel A), as confirmed also by histological analyses (Fig. 2, panel B, C and D).

Crypt destruction, and submucosal edema, including important necrosis of the epithelium, were more relevant in vehicle-treated animals (Fig. 2, C; c1, c2 and c3), with respect to MIA-690-treated animals, which also showed less lymphocyte infiltration (Fig. 2 Panel A and D; d1, d2, and d3). Furthermore, AOM/DSS induced an increase of 5-HT, PGE_2 and 8-iso-$PGF_{2\alpha}$ levels, which were significantly decreased in the AOM/DSS + MIA-690 group (Fig. 2 panel, E, F and G), suggesting that MIA-690 improved the general framework of the experimentally induced colitis.

At 5 weeks from the start of treatment with AOM/DSS, both groups (AOM/DSS + vehicle and AOM/DSS + MIA-690) developed colonic neoformations, possibly corresponding to tumors (Fig. 3, panel A). Macroscopical analyses showed a significant decrease not only on the number of tumors, but also on their distribution (Fig. 3, panel A) in mice treated with MIA-690 as compared to vehicle group (AOM/DSS + vehicle). The representative histological images of H&E-stained colon sections from each group are shown in Fig. 3, panel B and C.

The colon of MIA-690 treated mice showed focal mucosal hyperplasia, and in some areas low-grade inflammation (c1-c3), unlike the vehicle group that exhibited a high-grade dysplasia and tubular adenoma with scattered goblet cells (b1-b3). Gene expression of COX-2, TNF-α, NF-kB, iNOS, and IL-6 was significantly higher in both AOM/DSS groups, compared with control (untreated with AOM/DSS) (Fig. 3, panel D). However, the relative increase of all inflammatory markers was lower in mice treated with MIA-690, as compared to vehicle group. These data indicate that the tumor development induced by AOM/DSS treatment was significantly reduced by chronic treatment with MIA-690.

At 8 and 14 weeks from the start of treatment with AOM/DSS, the colon of mice treated with vehicle or MIA-690 macroscopically presented many aberrant crypts grouped in cluster (Fig. 4, panel A and B). However, the number of clusters and the percentage of the tissue involved were significantly lower in colon of mice treated with MIA-690. Treatment with MIA-690 did not completely abolish colon cancer, but the tumors appeared less aggressive, as demonstrated by histological analysis. In particular, the colons of animals treated with AOM/DSS + vehicle showed higher grade adenoma after 5 weeks of treatment (Fig. 3, b1-b3), while intramucosal carcinoma and invasive adenocarcinoma were evident at 8 and 14 weeks (Fig. 4, a1- a3 and b1- b3). In contrast, animals treated with MIA-690 developed only higher grade adenoma at 14 weeks of treatment with AOM/DSS (Fig. 4, b4-b6).

To further evaluate the role of MIA-690 in CRC progression, we analyzed by Western blot the expression of c-Myc, a central mediator of the oncogenic process underlying CRC development [19]. At 8 weeks, we observed an increase in c-Myc expression in colon of AOM/DSS treated animals with respect to untreated control mice (Fig. 4, panel C). However, chronic treatment of AOM/DSS mice with MIA-690 significantly reduced c-Myc expression as compared to AOM/DSS mice treated with vehicle (Fig. 4, panel C). The reduction of c-Myc expression was more marked after 14 weeks of treatment with MIA-690 than vehicle group (Fig. 4, panel C) (Supplementary Fig. S1).

3.2. MIA-690 induces growth inhibition in CRC by up-regulation of p53 and down-regulation of bcl-2 and P-AKT

To explore the mechanism underlying the CRC growth inhibition by treatment with MIA-690, we analyzed in colon tissues of AOM/DSS treated mice the protein expression levels of MAPK (mitogen-activated protein kinase), AKT (serine/threonine kinase), P-AKT (phosphorylated AKT), p53 and Bcl-2. It is well known that MAPK and AKT pathways play a key role in regulation of cell survival, proliferation, growth and apoptosis [20,21]. Western blot analysis of colon tissues showed no significant differences in MAPK protein levels among mice treated with vehicle and AOM/DSS mice treated with vehicle or MIA-690 (Fig. 5, panel A; Supplementary Fig. S2). AKT and P-AKT protein levels were significantly increased in AOM/DSS-treated groups as compared to controls (untreated with AOM/DSS) (Fig. 5, panel A; Supplementary Fig. S3 and S4). On the other hand, treatment with MIA-690 induced a significant decrease in the expression of P-AKT at 8 weeks of treatment (Fig. 5, panel A), as well as at 14 weeks, while no effects have been observed on AKT levels, compared with vehicle treated group (Fig. 5, panel B). Also, treatment with MIA-690 increased protein p53 and decreased bcl2 protein levels at 14 weeks, with respect to vehicle (Fig. 5, panel C and D; Supplementary Fig. S5 and S6).

4. Discussion

CRC is an aggressive tumor for which the existing treatment options, such as surgery, radiotherapy and neoadjuvant and palliative chemotherapies, show unsatisfactory long-term survival rates [1]. In the present study, we demonstrated that chronic treatment with MIA-690 promoted survival and gradually blunted tumor progression in experimentally induced colitis-associated cancer in mice, paralleled by reduced inflammation in colon tissue.

In our model of colorectal tumorigenesis induced by AOM/DSS, we observed a time-dependent increase of colitis DAI, and decreased body weight as well as higher mortality and survival rates worse with respect to control animals. However, chronic treatment with MIA-690 improves DAI score, and reduces loss of weight. In addition, treatment with MIA-690 significantly reduces mortality and improves the survival rates, as compared to vehicle-treated group. In this context, the overall mortality rate was reduced by 20% compared with control.

The potential beneficial effects induced by administration of MIA-690 could be related to decreased inflammation of colon, and improvement in the quality of life of our animals, as demonstrated by morphological and histological analysis. In particular, colon segments of mice treated with MIA-690 appeared less damaged macroscopically, and histological examination revealed only scant inflamed areas at 2 weeks of treatment. On the other hand, Jaszberenyi and collaborators demonstrated that MIA-690 prolonged the survival in transgenic mice (5XFAD strain by over 6 months) that develop neurodegenerative symptoms characteristic of human Alzheimer's disease [22]. In addition, recently we reported that chronic peripheral MIA-690 administration stimulated food intake and body weight, in mice [23]. Furthermore, MIA-690 decreased 5-HT, PGE_2 and 8-iso$PGF_{2\alpha}$ levels in AOM/DSS induced colitis-associated cancer, confirming our previous studies [8,9].

In the AOM/DSS murine model, the colitis-associated CRC develops through a multiple morphological progression. From the 4th-5th week onwards, foci of normal crypts develop into aberrant crypts. Thereafter, the latter progress into microadenoma, adenomatous polyps, and finally adenocarcinomas [12]. Generally, 3–10 macroscopic tumors develop in 80%− 100% of the animals, with tubular adenoma, dysplasia, and colitis with mucosal ulceration appearing from week 12 of the treatment

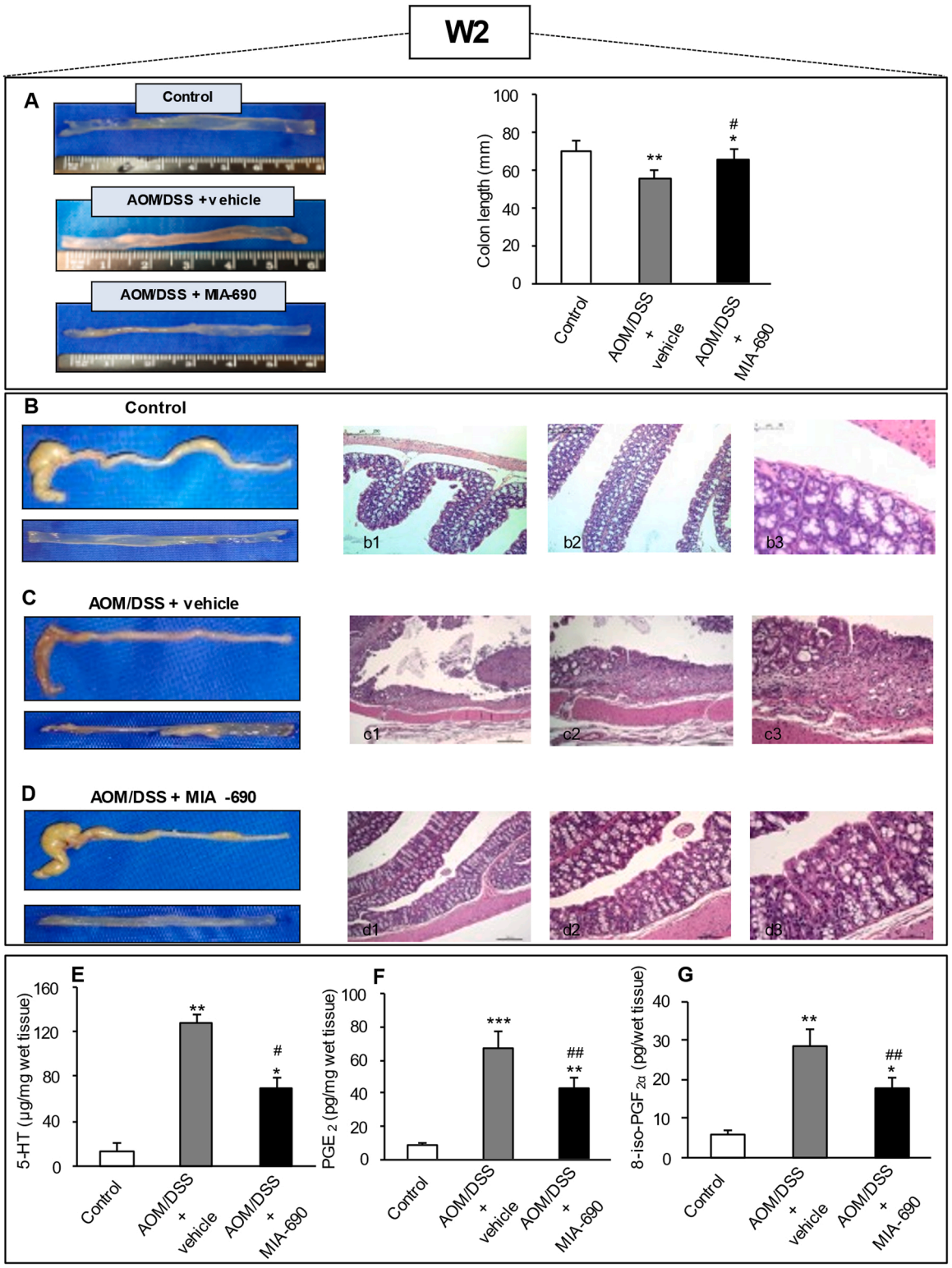

Fig. 2. Effect of s.c. administration of MIA-690 (5 µg) at W2 in AOM/DSS-treated mice. Colon segment length (A), macroscopical and histopathological changes (B, C and D), 5-HT, PGE_2 and 8-iso-$PGF_{2\alpha}$ levels (E, F and G) were determined at W2. B: normal colon segment (b1-b3); C (c1-c3) and D (d1-d3): inflammation of colon, necrosis of epithelium, distortion of crypts, epithelial erosion and immune cell infiltration in lamina propria and submucosa as well as submucosal edema. Hematoxilyn and eosin stain: the inserted bars indicate magnification. [H&E staining, x 5 (b1, c1, and d1); x 10 (b2, c2 and d2); x 20 (b3, c3 and d3) original magnification]. Data are expressed as means ± S.E.M. (n = 16–28 for each group); * $p < 0.05$, * * $p < 0.005$ and * ** $p < 0.001$ vs. control mice (untreated with AOM/DSS); $^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ vs. AOM/DSS + vehicle group.

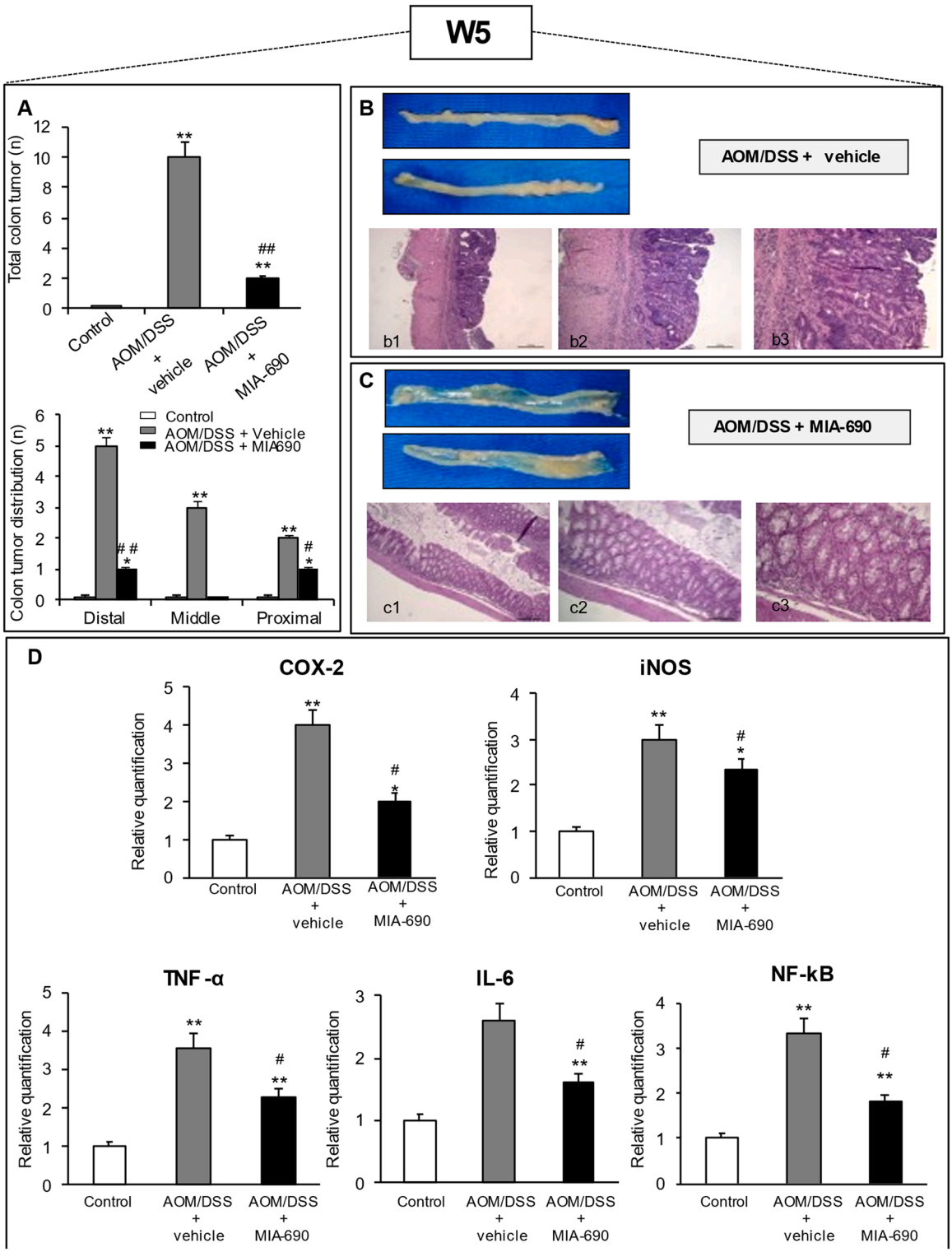

Fig. 3. Effect of s.c. administration of MIA-690 (5 μg) at W5 in AOM/DSS-treated mice. Total colon tumors (A), distribution of tumors in distal, middle and proximal tract (A), macroscopical and histopathological changes of colon (B and C), relative gene expression of COX-2, iNOS, TNF-α, IL-6 and NF-kB (D) on colon segment in a model of AOM/DSS-induced colorectal cancer. Presence of architectural alteration: high-grade dysplasia and tubular adenoma with scattered goblet cells (b1-b3); focal mucosal hyperplasia, and, in some areas, low-grade inflammation (c1-c3). Hematoxilyn and eosin stain: the inserted bars indicate magnification. [H&E staining, x 5 (b1, c1); x 10 (b2, c2); x 20 (b3, c3) original magnification]. Data are expressed as means ± S.E.M. (n = 16–28 for each group); * $p < 0.05$ and * * $p < 0.005$ vs. control mice (untreated with AOM/DSS); $^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ vs. AOM/DSS + vehicle group.

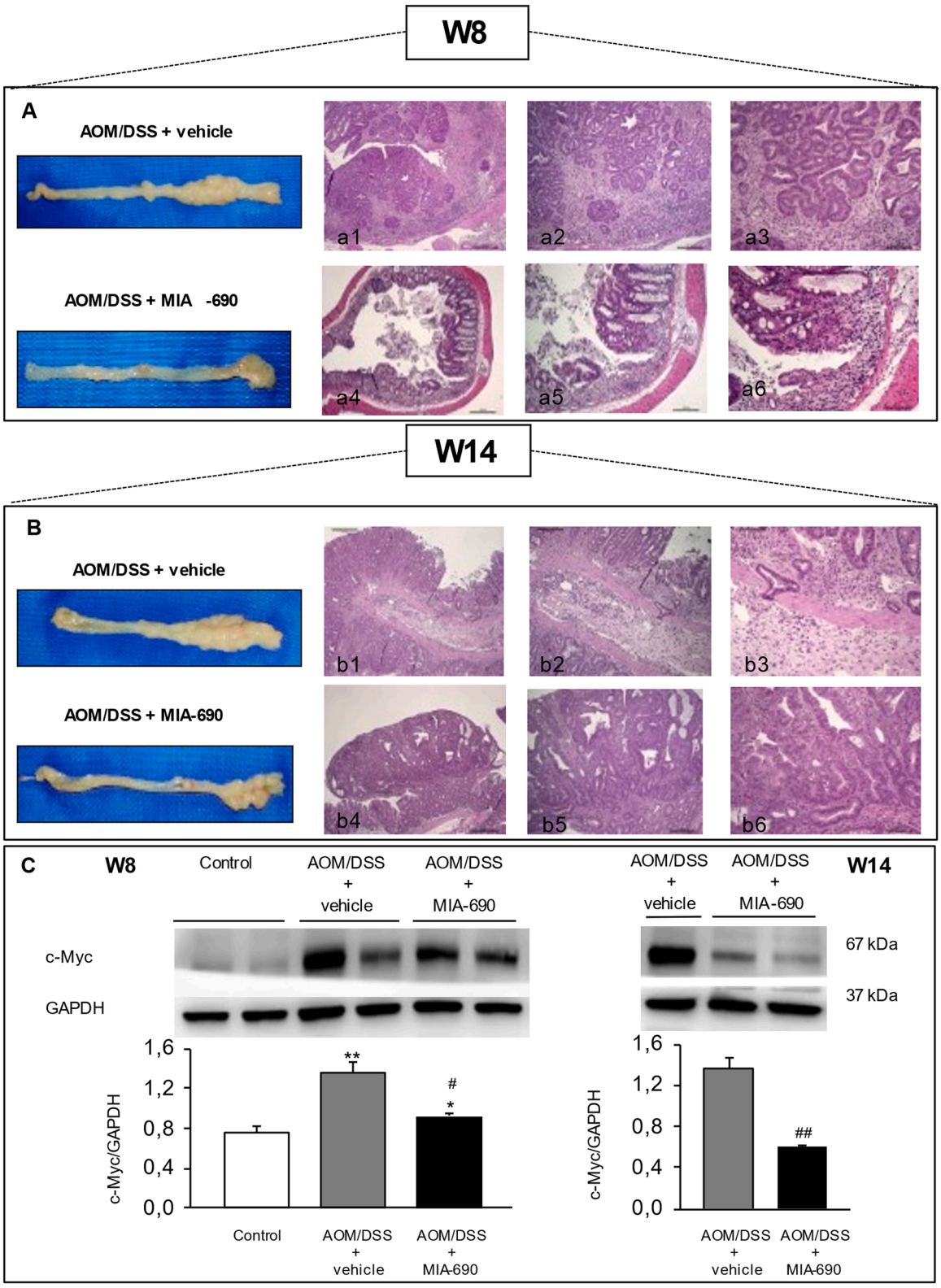

Fig. 4. Effect of s.c. administration MIA-690 (5 μg) at W8 and W14 in AOM/DSS-treated mice. Macroscopical and histopathological changes of colon (A and B), protein expression of c-Myc (D) on colon segment in a model of AOM/DSS-induced colorectal cancer. Presence of intramucosal carcinoma and invasive adenocarcinoma at W8 and W14 (a1-a3 and b1-b3); high grade adenoma at W14 of treatment with AOM/DSS (b4-b6). Hematoxilyn and eosin stain: the inserted bars indicate magnification. [H&E staining, x 5 (a1-a4, b1-b4); x 10 (a2-a5, b2-b5); x 20 (a3-a6, b3-b6) original magnification]. Western blot for c-Myc (67 kDa) protein in which GAPDH (36 kDa) was used as a loading control. Data are expressed as means ± S.E.M. (n = 16–28 for each group); * $p < 0.05$ and * * $p < 0.005$ vs. control mice (untreated with AOM/DSS); $^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ vs. AOM/DSS + vehicle group.

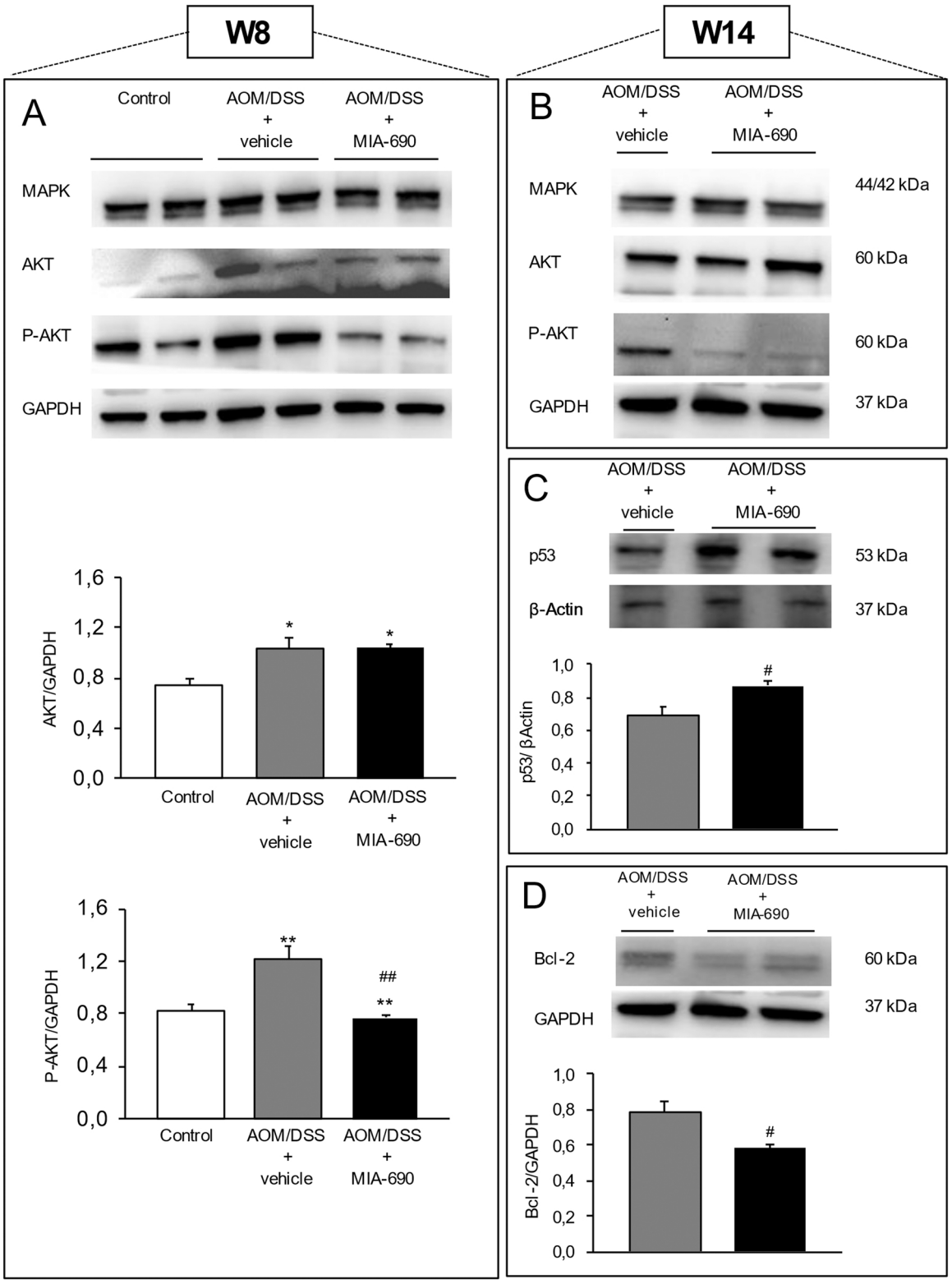

Fig. 5. Effect of s.c. administration MIA-690 (5 μg) at W8 and W14 on p53, bcl-2 and P-AKT protein expression. Examples of western blot of MAPK (44/42 kDa) (A and B), AKT (60 kDa) (A and B), P-AKT (60 kDa) (A and B), bcl-2 (60 kDa) (D) proteins in which GAPDH (36 kDa) was used as a loading control. Western blot of p53 (53 kDa) (C) protein in which β-actin was used as a loading control. Data are expressed as means ± S.E.M. (n = 16–28 for each group); * $p < 0.05$ and * * $p < 0.005$ vs. control mice (untreated with AOM/DSS); $^{\#}p < 0.05$ vs. AOM/DSS + vehicle group.

[12]. In our experiments, macroscopic and histological examination demonstrated many aberrant crypts and different grades of dysplasia, since week 5.

However, chronic treatment with MIA-690 significantly decreased the number of neoformations and their distribution in colon tissue compared with animals treated with vehicle. Moreover, unlike the vehicle group that showed dysplasia of higher grade, the animals treated with MIA-690 showed only mild hyperplasia and low-grade

inflammation.

In our model of colorectal tumorigenesis, MIA-690 might have slowed down cancer progression by reducing various inflammatory and oxidative markers. It is well known that chronic colon inflammation represents a fundamental prerequisite for the onset of carcinogenesis [24]. In this context, the inflammatory cytokines and oxidative/nitrosative DNA damage play a relevant role in inflammation-related carcinogenesis [24]. As for human CRC, AOM/DSS-induced tumors show increased levels of enzymes involved in synthesis of prostaglandin E_2 and nitric oxide, such as COX-2 and iNOS, as well as NF-kB, TNF-α, and IL-6 [25]. On the other hand, various reports demonstrated that levels of PGE_2, COX-2, NF-kB, TNF-α, IL-6, iNOS, and 8-iso-$PGF_{2\alpha}$ are closely related to cancer development and tumor progression [26–30]. In addition, the expression of COX-2 is usually increased in inflammation, as well as in 40% of colorectal adenomas and 80% of CRCs [31]. Actually, COX-2 plays an important role in apoptosis, angiogenesis, and tumor invasiveness, as well as in the progression of cancer [32]. Moreover, mRNA and protein levels of TNF-α, a key regulator of inflammation, are drastically increased in the preneoplastic inflamed colonic mucosa, as well as in tumor formation [30]. Furthermore, TNF-α and NF-kB affect tumor number and size in CRC [28,30].

MIA-690 significantly decreased COX-2, iNOS, TNF-α, IL-6 and NF-kB gene expression as compared to vehicle. On the other hand, different GHRH antagonists were shown to suppress the expression of inflammatory genes in prostatic hyperplasia and breast cancer [33,34]. In addition, MIA-690 decreased various inflammatory and oxidative markers, such as COX-2, TNF-α, NF-kB and iNOS, in *ex vivo*, as well as in *in vivo* studies [8,9].

CRC progression has also been finally evaluated at 8 and 14 weeks. Fig. 4 shows various neoformations, grouped in cluster, in distal colon, as well as the presence of several high-grade adenoma, intramucosal carcinoma and invasive adenocarcinoma in AOM/DSS + vehicle-treated animals at 8 and 14 weeks, respectively. Chronic treatment with MIA-690 delayed cancer progression, inducing hyperplasia and marked inflammation at 8 weeks and high-grade adenoma, without invasion of sub-mucosa, at 14 weeks (Fig. 4, panel B). MIA-690 might have slowed down cancer progression not only by reducing various pro-inflammatory and oxidative markers, but also inhibiting the expression of c-Myc, a well-known protoncogene (Fig. 4, panel C).

Similarly to human CRC, AOM/DSS-induced tumors show a dysregulation of some genes, including target genes of APC/β-catenin signaling pathway, represented by c-Myc [35]. c-Myc functions as a central mediator of the oncogenic process underlying development of CRC [19]. In accordance with the literature, we observed elevated expression of c-Myc in AOM/DSS-treated colon compared with normal tissue. By contrast, MIA-690 significantly reduced c-Myc, at both 8 and 14 weeks, with greater effect at 14 weeks. Numerous lines of evidence indicate that decreased c-Myc expression leads to reduced numbers of CRCs [36], a result confirmed by c-Myc in human CRC [37]. Further, Villanova and collaborators reported that chronic administration of MIA-690 (5 μg/day) strongly inhibited the growth of malignant pleural mesothelioma, also reducing c-Myc levels [38].

MAPK pathways regulate many cellular functions including cell proliferation, differentiation, migration and apoptosis [20]. It has been demonstrated that GHRH stimulates the proliferation of cancer cells through MAPK [39,40] and GHRH antagonists suppress cell proliferation through the signaling pathways MAPK/ERK(1/2) and/or P13K/Akt [39,40]. We did not find any effect of AOM/DSS treatment on MAPK protein expression at both 8 and 14 weeks (Fig. 5, panel A). This discrepancy could be due to the experimental model used. In particular, Tang and collaborators demonstrated that MAPK signaling was upregulated in the inflammatory and low-grade dysplastic phases but returned to normal level in the high-grade dysplastic and cancerous phase [41].

We also observed that the treatment with AOM/DSS induced overexpression of AKT and P-AKT at both 8 and 14 weeks. The AKT serine/threonine kinase, as well as P-AKT, play a key role in regulation of cell survival, proliferation, growth and apoptosis [21]. On the other hand, the overactivation of AKT and P-AKT is a common molecular characteristic of multiple malignancies [42], representing a possible target for cancer prevention and therapy. MIA-690 decreased P-AKT protein expression as compared to vehicle group. On the other hand, Guo and collaborators demonstrated that treatment with an early GHRH antagonist JMR-132 induced a down-regulation of P-AKT, without modifying the expression of AKT, in ovarian cancer cells [43]. Finally, p53 upregulation and Bcl-2 downregulation induced by MIA-690 at 14 weeks could partially explain the effects of MIA-690 in colitis-associated CRC. p53 and Bcl-2 play a pivotal role in the regulation of cell death, while P-AKT controls cell cycle, invasion and migration [21,44]. We found that MIA-690 could promote the apoptosis of colon cancer cells by regulating the expression of p53 and Bcl-2, through binding with pGHRH-R. MIA-690 displayed potent inhibitory actions in malignant tumors, such as pleural mesothelioma [38], retinoblastoma [45], melanoma [46], as well as on lung and prostate cancers [47,48]. In addition, MIA-690 shows a stronger inhibitory effect in HCT-15 cells with respect to other GHRH antagonists, with a decrease of cell growth by 40% [49] through binding to pGHRH-R [50]. Schematic representation of the possible antitumor effect of MIA-690 in CRC is shown in Fig. 6. In addition, a balance between p53 and MAPK pathways has been previously found to play a key role in preventing or promoting initiation of tumours. P53 has also been suggested to exert anti-inflammatory activities [51,52], by down-regulating iNOS and COX-2 [53]. In this context, we can speculate that the inhibitory effects of MIA-690 on gene expression of iNOS and COX-2 could be related, at least in part, to p53.

MIA-690 slows down colorectal cancer (CRC) progression and growth by reducing inflammatory and oxidative markers and modulating apoptotic and oncogenic pathways involved in the survival, proliferation and growth of experimental CRC.

It is important to stress that we tested the effects of MIA-690 after treatment with AOM/DSS for 14 weeks. To further clarify the role of MIA-690 in colorectal tumorigenesis, it would be interesting to study its preventive effects in the same experimental model. In conclusion, MIA-690 inhibited the inflammation, progression and growth of experimental CRC, prolonging survival in tumor-bearing mice. Further investigations are required for translating these findings into the clinics.

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CRediT authorship contribution statement

Lucia Recinella: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **Annalisa Chiavaroli:** Formal analysis, Investigation, Project administration, Supervision, Validation. **Serena Veschi:** Formal analysis, Investigation, Project administration, Supervision. **Valentina Di Valerio:** Formal analysis, Investigation, Project administration, Supervision. **Rossano Lattanzio:** Formal analysis, Investigation, Project administration, Supervision. **Giustino Orlando:** Data curation, Software, Visualization. **Claudio Ferrante:** Data curation, Software, Visualization. **Iacopo Gesmundo:** Formal analysis, Investigation, Validation. **Riccarda Granata:** Formal analysis, Supervision, Writing – review & editing. **Renzhi Cai:** Resources. **Wei Sha:** Resources. **Andrew V. Schally:** Resources, Supervision, Writing – review & editing. **Luigi Brunetti:** Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Sheila**

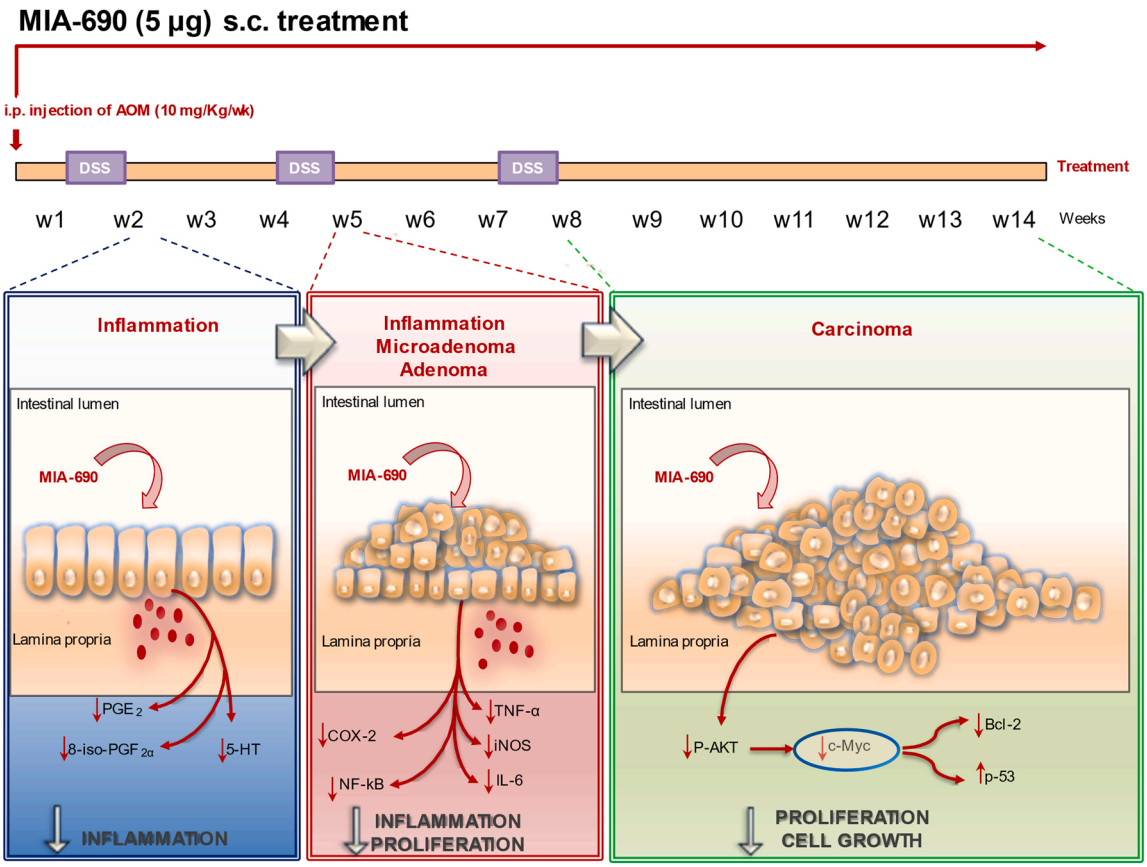

Fig. 6. Schematic representation of the antitumor effect of MIA-690 in CRC.

Leone: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

A.V.S. and R.C. are listed as co-inventors on patents for GHRH antagonists, assigned to the University of Miami, Miami, FL, and the Veterans Affairs Medical Center, Miami. L.R., A.C., G.O., C.F., S.V., V.D., R. L., I.G., R.G., W.S., L.B. and S.L. declare no potential conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112554.

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