Supplemental materials and methods


Inhibition of colon carcinogenesis by 2-methoxy-5-amino-N-hydroxybenzamide, a novel derivative of 5-aminosalicylic acid

Isolation and culture of intestinal fibroblasts and intra-epithelial lymphocytes (IELs)

Intestinal fibroblasts were isolated from normal colonic specimens of 3 patients who underwent colectomy for colon cancer and cultured as described elsewhere. All reagents were from Sigma-Aldrich (Milan, Italy), unless specified. CFSE-labelled fibroblasts were cultured with or without 2-14 for 48 hours and then analysed by flow cytometry. Normal colonic IELs were isolated as described elsewhere, labelled with CFSE and either left unstimulated or stimulated with anti-CD3/CD28-bound beads (Miltenyi Biotec, Calderara di Reno, Italy) in the presence or absence of 2-14 for 48 hours. The percentage of proliferating cells was evaluated by flow cytometry.

RNA extraction, cDNA preparation and Real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen), retro-transcribed into complementary DNA (cDNA), and amplified using the following conditions: denaturation 1 minute at 95°C, annealing 30 seconds at 60°C for cyclin D1 or 30 seconds at 62°C for β-actin, followed by 30 seconds of extension at 72°C. Primers sequence of cyclin D1 was: FWD: 5´-AGGCGGAGGAGAACAAACAG-3´; REV: 5´-CGGTAGTAGGACAGGAAGTTG-3´. Real-time PCR was performed using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy). β-actin (FWD: 5´-AAGATGACCCAGATCATGTGTTTGAGACC-3´ and REV:5´-AGCCAGTCCAGACGCAGGAT-3)
was used as an internal control. Activating transcription factor 4 (ATF4) RNA expression was evaluated using a Taqman assay (Applied Biosystems, Milan, Italy). RNA expression was calculated relative to the housekeeping β-actin gene on the base of the ddCt algorithm.

**XBP-1 RT-PCR**

Amplification of the unspliced and spliced forms of x-box-binding protein-1 (XBP-1) RNA was performed as described elsewhere, using the following primers: 5′-GTTGAGAACCAGGAGTTAAGACAG-3′ (sense) and 5′-CAGAGGGATATCTCAAGACTAGG-3′ (antisense). GAPDH primers were as follows: 5′-CTCAGACACCATGGGGAAGGTGA-3′ (sense) and 5′-ATGATCTTGAGGCTGTGTCATA-3′ (antisense).

**Effect of 2-14 on the in vivo formation of CT26-derived tumors**

CT26 cells (1 x 10^5 cells in 300 μl PBS 1X) were injected subcutaneously into the flank of Balb/c mice, whose fur was shaved and depilated. Five groups of 10 mice each were implanted with CT26. The first group received daily subcutaneously 300μl PBS 1X (control), the remaining groups received daily subcutaneously 2-14 at a final dose of 1, 4, 8, or 12 mg/kg/mouse dissolved in 300μl PBS 1X. Both 2-14 and PBS were administered starting on day 3. In further experiments, mice were injected with PBS or 2-14 (8 mg/kg/mouse) intraperitoneally (i.p.). Mice were monitored over the time for weight loss and sacrificed at day 14. Tumors were photographed, excised, and their volume calculated, assuming it is an ellipsoid, using to the following formula: 1/2 x (short diameter) x (long diameter) x (height). Proteins extracted from xenografts were analysed for cyclin D1 expression by Western blotting. To examine whether 2-14 reduced the growth of established CRC xenografts, Balb/c mice were implanted with CT26 as described above. After 2 weeks, mice with similar tumor volume, determined by caliper measurements, were divided into three groups of 8 mice each. The first group
received daily subcutaneously 300 μl PBS (control), the second group received daily subcutaneously 5-ASA (80 mg/kg) dissolved in 300 μl PBS. The last group received daily subcutaneously 2-14 (8 mg/kg) dissolved in 300 μl PBS. After 2 weeks, mice were sacrificed, tumors were photographed, then excised and their volume calculated as described above. Studies were approved by the Local Ethical Committee.

**Induction of colitis-associated colon cancer**

Balb/c mice were given a first i.p. injection of azoxymethane (AOM: 10 mg/kg) on day 0. Seven days after the AOM injection, the mice were given 2% dextran sulfate sodium (DSS; mw: 9,000-20,000) in the drinking water for 7 days. One week after the discontinuation of DSS administration, mice received a second i.p. injection of AOM (5 mg/kg). Then, 7 days after the second AOM injection, mice were again given 2% DSS in the drinking water for 7 days. Two weeks later, the mice were randomly divided into two groups receiving i.p. injection of PBS or 16 mg/kg/mouse 2-14 every second day, for 49 days until sacrifice. Mice were endoscopically screened one week prior to being sacrificed using the high resolution endoscopic system and protocols described by Becker *et al* [4]. Neoplastic lesions were graded, scored and summed to yield the tumor score as previously described [4]. All mice were sacrificed at the end of the study, and colonic samples were used for histological analysis and isolating lamina propria mononuclear cells (LPMC).

**LPMC isolation**

LPMC were isolated from freshly obtained colons as described elsewhere [5]. Briefly, the entire colons from each group were longitudinally cut, washed in Hank’s balanced salt solution (HBSS)-calcium-magnesium free (Lonza), finely minced and incubated in HBSS containing 5 mM Ethylenediamine-tetraacetic acid (EDTA), 145 mg/ml dithiothreitol, 1M Hepes (Lonza), 10% fetal
bovine serum, and 1% penicillin/streptomycin at 37°C for 15 min for two cycles. EDTA was then removed by 3 washes in HBSS, and the tissue was digested in RPMI 1640 containing 400 ng/ml collagenase D (cat. 11088882001, Roche Diagnostic, Mannheim, Germany) and 0.01 mg/ml DNase I (cat. 10104159001, Roche Diagnostic) in a shaking incubator at 37°C. The resulting supernatants were then resuspended in 20ml of 30% Percoll and centrifuged to isolate the LPMC-enriched population. An aliquot of LPMC was assessed for the expression of CD4, CD8, and CD25 by flow cytometry using the following antibodies: CD4, cat. 552051, BD Pharmigen, Milan, Italy; CD8, cat. 22150084S and CD25, cat. 22150253, Immunotools, Friesoythe, Germany). The remaining LPMC were used for extracting RNA and assessing the expression of cyclooxygenase (COX)-2, Tumor Necrosis factor (TNF)α, and interleukin (IL)-1β by real-time PCR. To this end, the following primers were used: COX-2: FWD: 5´-TTCTTTGCCACCAGCTTCAC-3´; REV: 5´-GGATACACCTCTCCACCAAT-3´, TNFα: FWD: 5´-ACCCTCACACTCAGATCATC-3´; REV: 5´-GAGTAGACAAGGTACAACCC-3, IL-1β: FWD: 5´-TCAGGCAGGCAGTATCACTC-3´; REV: 5´-CTAATGGGAACGTCACACACC-3.

**Histological analysis and immunohistochemistry**

Colonic sections were stained with hematoxylin and eosin (H&E). The degree of inflammation and the presence of neoplastic lesions were evaluated blindly by a single experienced gastrointestinal pathologist (GP) as described previously. Lesions were classified as negative or positive for dysplasia/carcinoma, using the criteria that are used in the daily practice of clinical surgical pathology. A diagnosis of carcinoma was performed when neoplastic glands had invaded beyond the muscularis mucosae and into the submucosa. Any dysplasia or cancer that had an elevated growth pattern if seen grossly or microscopically was considered to be a polypoid lesion. Flat lesions had no elevated component. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed using
a PCNA staining kit (ZYMED Laboratories, Carlsbad, CA, USA) according to the manufacturer’s instructions.
References (to supplemental materials and methods)

Supplemental Figures

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Suppl. Figure 1

Chemical structure of 5-ASA and 5-ASA derivatives tested in the study.
Suppl. Figure 2

2-14 is more potent than 5-ASA and other 5-ASA derivatives in inhibiting CRC cell growth.

Percentage of growth inhibition, assessed by flow cytometry, of CFSE-labeled HCT-116 cells, following treatment with increasing doses of 5-ASA or 5-ASA-derived compounds for 24 hours. Data indicate mean ± SD of 3 experiments. (* P<0.001; ** P<0.01).
Suppl. Figure 3

Anti-proliferative effects of 2-14 on CT26, a murine CRC cell line.

(A) 2-14 dose-dependently inhibits the growth of CT26 cells. CFSE-labeled cells were either left untreated (Untr) or treated with increasing doses of 2-14. After 24 hours, the percentage of proliferating cells was evaluated by flow cytometry. Data indicate mean ± SD of 3 experiments. (untreated vs 2-14-treated cells * P=0.01; ** P<0.001).

(B) 2-14 induces CT26 cells to accumulate in G0/G1 phase. Cells were either left untreated (Untr) or treated with 2-14 for 24 hours, and the cell cycle distribution was analyzed by flow cytometry. Values are the percentages of cells in the different phases of cell cycle and indicate mean ± SD of 4 experiments. A significant increase in the number of cells that accumulate in G0/G1 phase is seen after 2-14 treatment (untreated vs 2-14-treated cells * P=0.03; ** P<0.01).

(C) 2-14 enhances the phosphorylation of eIF2α at Ser51 residue and down-regulates cyclin D1 protein expression. Cells were either left untreated (Untr) or treated with 1.5 mM 2-14 for 6 and 12 hours, and protein extracts were then evaluated by Western blotting. One of 3 representative Western blots is shown.
Suppl. Figure 4

Administration of 2-14 in mice does not alter the type of mucosal lymphocytic infiltrate and the RNA content for inflammatory markers.

The anti-neoplastic effect of 2-14 in the AOM/DSS-induced colon cancer model is associated with no change in the percentages of CD4+, CD8+, and CD25+ cells infiltrating the colonic lamina propria (A), and in the RNA content for COX-2, TNF-α, and IL-1β in colonic LPMC (B).