Inhibition of Colon Carcinogenesis by 2-Methoxy-5-Amino-N-Hydroxybenzamide, a Novel Derivative of Mesalamine

CARMINE STOLFI,* MASSIMILIANO SARRA,* ROBERTA CARUSO,* MASSIMO CLAUDIO FANTINI,* DANIELE FINA,* ROBERTO PELLEGRINI,* GIAMPIERO PALMIERI,‡ THOMAS T. MACDONALD,§ FRANCESCO PALLONE,* and GIOVANNI MONTELEONE*

*Department of Internal Medicine, ‡Pathological Anatomy Unit, University of Tor Vergata, Rome, Italy; and §Institute of Cell and Molecular Science, Bart's and the London School of Medicine and Dentistry, London, UK

BACKGROUND & AIMS: Mesalamine has been reported to protect against inflammatory bowel disease–related colorectal cancer (CRC), but several drug-related issues have limited its use in chemopreventive programs. We evaluated the antineoplastic properties of mesalamine derivatives using in vitro and in vivo models of CRC. **METHODS:** CRC cell proliferation and cell-cycle progression were evaluated by flow cytometry after exposure to mesalamine or mesalamine derivatives. Cyclins, cyclin-dependent kinases, and endoplasmic reticulum stress–related molecules were examined by immunoblotting. The in vivo antineoplastic effect of 2-methoxy-5-amino-N-hydroxybenzamide (2-14) was evaluated in a syngenic, CT26-derived xenograft mouse model of CRC and in the azoxymethane/dextran sulfate sodium–induced mouse model of colitis-associated CRC. **RESULTS:** The mesalamine derivative 2-14 was 10-fold more potent than mesalamine in inhibiting CRC cell proliferation. After exposure to 2-14, cyclin D1 expression was reduced and G0/G1 phase cells accumulated. These events were preceded by activation of eukaryotic translation initiation factor 2-alpha kinase 3 (pancreatic endoplasmic reticulum eIF2α kinase), phosphorylation of eukaryotic translation initiation factor 2alpha, induction of activating transcription factor 4, and splicing of X-box binding protein 1 messenger RNA, events that define endoplasmic reticulum stress. Silencing of PERK restored cyclin D1 levels, allowing cells to overcome the cell-cycle block induced by 2-14. Mice injected with 2-14 developed fewer CRC xenograft-derived tumors. Moreover, 2-14 injection reduced the development of neoplastic lesions induced by azoxymethane and dextran sulfate sodium in mice. **CONCLUSIONS: The mesalamine derivative 2-14 inhibited CRC cell proliferation in vitro and prevented CRC progression in mouse models.**

Colorectal cancer (CRC) is a common malignancy in the Western world. Despite recent advances in CRC pathogenesis and therapy, this neoplasia is still one of the leading causes of cancer-related deaths.[1] This suggests the necessity of effective chemopreventive/chemotherapeutic agents.

Several epidemiologic and experimental studies have shown that intake of nonsteroidal anti-inflammatory drugs reduces the risk of developing CRC.[2,3] However, the wide use of nonsteroidal anti-inflammatory drugs in the chemoprevention of CRC has been limited by their frequent and often severe gastrointestinal side effects. Even the use of the more selective inhibitors of cyclooxygenase-2 partly has been hampered by serious drug-related complications.[4]

Promising data on CRC chemoprevention recently have emerged from epidemiologic studies conducted in patients with inflammatory bowel disease (IBD), a clinical condition that is associated with enhanced CRC risk. It was reported that mesalamine or mesalazine, the drug of choice in the maintenance of remission, and treatment of mild flare-ups of IBD, can reduce the incidence of IBD-related CRC.[5–7] Experimental studies showed also that mesalamine can inhibit biological pathways that sustain CRC cell growth.[8–10] However, some drug-related issues have limited the use of mesalamine in the chemopreventive programs. In fact, most of the in vitro anticancer effects of mesalamine are seen with doses of the drug that are not always reached within the gut tissue under standard oral treatment.[11] Moreover, mesalamine is inactivated rapidly and eliminated from the circulation when given systemically,[12,13] and orally administered mesalamine does not reach the left colon, where many sporadic and colitis-related CRCs develop. Finally, it is noteworthy that some epidemiologic studies failed to confirm the chemopreventive activity of mesalamine.[14,15] Therefore, the validation of novel mesalamine derivatives that show similar safety profiles but enhanced anticancer activity would be highly desirable.

We have developed several structurally related mesalamine compounds, and initially assessed their effect

Abbreviations used in this paper: 2-14, 2-methoxy-5-amino-N-hydroxybenzamide; CDK, cyclin-dependent kinase; CRC, colorectal cancer; DSS, dextran sulfate sodium; eIF2, eukaryotic initiation factor 2; ERS, endoplasmic reticulum stress; IBD, inflammatory bowel disease; PERK, pancreatic ER eIF2α kinase; PI, propidium iodide; siRNA, short interfering RNA; UPR, unfolded protein response.

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on the in vitro proliferation of CRC cells. Among these derivatives, we selected the compound 2-methoxy-5-amino-N-hydroxybenzamide (herein termed *2-14*) because it displayed a more pronounced antimitogenic effect and was 10 times more potent than mesalamine in blocking CRC cell growth. Next, we explored the molecular mechanisms underlying the antineoplastic action of 2-14, and evaluated its anticancer activity in vivo.

Materials and Methods

Cell Proliferation

All reagents were from Sigma-Aldrich (Milan, Italy) unless specified. Mesalamine (Giuliani SpA, Milan, Italy) and mesalamine derivatives were dissolved as a 100-mmol/L or 25-mmol/L stock solution, respectively, in culture medium. The pH of each drug solution was adjusted to 7.4 with NaOH if necessary. The human CRC cell lines, HCT-116 and HT-29, were maintained in McCoy's 5A medium and the murine CRC cell line, CT26, was maintained in RPMI 1640 medium, both supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Lonza, Verviers, Belgium). For cell growth assays, cells were labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Milan, Italy) at 37°C for 30 minutes. The medium then was removed, and fresh media containing 0.05% bovine serum albumin and increasing doses of mesalamine or mesalamine derivatives were added. After 24 hours cells were incubated with 5 μg/mL of propidium iodide (PI) for 15 minutes, at 4°C. Carboxyfluorescein diacetate succinimidyl ester and/or PI-positive cells were determined by flow cytometry.

Cell Cycle

For analysis of cell-cycle distribution, cells were cultured in medium containing 0.05% bovine serum albumin and increasing doses of 2-14 for 24 hours, pulsed with 10 μmol/L bromodeoxyuridine for 60 minutes, fixed in 70% cold ethanol, and stored at −20°C for at least 3 hours. Cells then were denatured in 2 mol/L HCl, and stained with anti–bromodeoxyuridine monoclonal antibody (Immunotech, Marseille, France) followed by fluorescein isothiocyanate–conjugated secondary anti-mouse immunoglobulin G (Molecular Probes, Milan, Italy). After staining with 100 μg/mL PI, cells were analyzed by flow cytometry.

Western Blotting

Total proteins were extracted and analyzed by Western blotting as described elsewhere.[16] Blots then were incubated with the following antibodies: cyclin D1 (sc-20044), cyclin D2 (sc-53637), cyclin D3 (sc-182), cyclin E (sc-25303), cyclin-dependent kinase (CDK)4 (sc-601), CDK6 (sc-177), p-CDK2 (T14-Y15) (sc-28435-R), CDK2 (sc-6248), eukaryotic initiation factor 2 (eIF2)α (sc-11386), pancreatic endosplasmic reticulum eIF2α kinase (PERK, sc-13073) (all from Santa Cruz Biotechnology, Santa Cruz, CA), p-eIF2α Ser51 (Cell Signaling, DBA Italia, Milan, Italy), and p-PERK Thr980 (Biolegend, San Diego, CA), followed by a secondary antibody conjugated to horseradish peroxidase. To ascertain equivalent loading of the lanes, each blot was stripped and incubated with a mouse–anti-human monoclonal β-actin antibody.

Isolation and Culture of Intestinal Fibroblasts and Intraepithelial Lymphocytes

Please see Supplementary Materials and Methods section.

PERK Knockdown by Short Interfering RNA

HCT-116 were transfected with pancreatic endoplasmic reticulum eIF2 α kinase (PERK) or control short interfering RNA (siRNA) (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen). The efficiency of the siRNA knockdown was assessed using fluorescein-conjugate control siRNA (Santa Cruz Biotechnology).

Analysis and Quantification of Cell Death and Caspase-3 Activation

To assess cell death, cells were treated with 2-14 (1.5–3 mmol/L) in the presence or absence of Q-VD-OPh, a pan-caspase inhibitor (R&D Systems, Inc, Minneapolis, MN). After 16–48 hours, cells were stained with fluorescein isothiocyanate–Annexin V (Becton Dickinson, Milan, Italy), incubated with PI (5 μg/mL) for 30 minutes at 4°C, and analyzed by flow cytometry. Caspase-3 activation was quantified by flow cytometry using the CaspGLOW staining kit (Biovision Research Products, Mountain View, CA).

RNA Extraction, Complementary DNA Preparation, and Real-Time Polymerase Chain Reaction

Please see Supplementary Materials and Methods section.

XBP-1 Reverse Transcription Polymerase Chain Reaction

Please see Supplementary Materials and Methods section.

Effect of 2-14 on the In Vivo Formation of CT26-Derived Tumors

Please see Supplementary Materials and Methods section.

Induction of Colitis-Associated Colon Cancer

Please see Supplementary Materials and Methods section.

Lamina Propria Mononuclear Cells Isolation

Please see Supplementary Materials and Methods section.