Appendix e2 Analytics for γH2AX assay

Analysis of H2AX was blinded: the personnel of the analytical laboratory were not aware of the subject, the anatomical location, or to which study visit the sample related. At the end of the study, the unique biopsy identification numbers were reconciled with patients’ identification using the investigator’s decoding list.

Analysis of γH2AX was performed as previously recommended [14, 15]. In order to obtain a single cell suspension, individual biopsies were incubated in 100 units/mL collagenase V for 2 hours at 37 °C with gentle rotation. The cells were then washed once with phosphate buffered saline (PBS), then incubated in 0.1 % saponin/PBS for 1 hour at room temperature to permeabilize them prior to antibody incubation. Antibody to γH2AX was then added and the cells were incubated for 1 additional hour. Antibody-stained cells were washed with PBS and then resuspended in 200 µL of PBS/2 % bovine serum before being run through a flow cytometer.

All biopsy samples from each patient were processed and analyzed on the same day, along with the negative and positive controls.

Assay controls
The negative and positive controls were represented by a human colonic epithelial cell line stained for γH2AX after treatment with saline or a DNA-damaging compound, respectively. Because the antibody is conjugated with a fluorophore (fluorescein isothiocyanate [FITC]), cells that contain DNA double-strand breaks will show higher relative fluorescence intensity than those with no or undetectable levels of DNA damage. Negative and positive (etoposide-treated) control cells were run concurrently to ensure proper assay execution.

Appendix e1 Inclusion/exclusion criteria

Inclusion criteria
1 Age: 18 – 75 years inclusive.

2 Indication: outpatients scheduled for screening or surveillance colonoscopy identified as having the clinical requirement for a second colonoscopy within 2 weeks of the initial colonoscopy.

3 Contraception: using at least one reliable method of contraception or abstinence for the duration of the study for women of child-bearing potential; or being of non-child-bearing potential or post-menopausal status for at least 1 year; negative pregnancy at screening for all women.

4 Full comprehension: ability to comprehend the full nature and purpose of the study, including possible risks and side effects; ability to cooperate with the investigator and to comply with the requirements of the entire study.

5 Informed consent: signed written informed consent before inclusion in the study.

Exclusion criteria
1 Pregnancy: pregnant or lactating women or women undergoing fertility treatment.

2 Physical findings: clinically significant abnormal physical findings that could interfere with the objectives of the study.

3 Laboratory analyses: clinically significant abnormal laboratory values indicative of physical illness; in particular, ALT, AST, γ-GT, bilirubin, creatinine, or urea greater than 2.5 × the upper limit for normal, based on local laboratory testing.

4 Allergy: ascertained or presumptive hypersensitivity to methylene blue and/or ingredients of both methylene blue MMX tablets and polyethylene glycol (PEG)-based bowel cleansing preparation; history of anaphylaxis to drugs or allergic reactions in general, which the investigator considered might affect the outcome of the study.

5 Disease: known or suspected gastrointestinal obstruction or perforation, toxic megacolon, major colonic resection, severe diverticulosis with diverticulitis, heart failure (Class III or IV), serious cardiovascular disease, severe liver failure, end-stage renal insufficiency, clinical alarm symptoms or history of anemia (Hb < 10 mg/dL), frank blood in the stool within the last 30 days prior to enrolment, known deficiency of glucose-6-phosphate dehydrogenase, known deficiency of NADPH reductase, methemoglobinemia, and any other medical condition that would have made the administration of the study drug or procedures hazardous to the subject.

6 Medication: previous or concomitant treatment with any monoamine oxidase inhibitor in accordance with a drug safety alert published by FDA [12]. In particular, previous or concomitant treatment with the selective serotonin reuptake inhibitors, the serotonin – norepinephrine reuptake inhibitors, tricyclic antidepressants, and other psychiatric drugs within 2 weeks before the study, previous or concomitant treatment with fluoxetine within 5 weeks before the study, and/or previous or concomitant treatment with anticoagulants or anti-aggregants inducing an INR > 1.5.
For the controls, immortalized human colonic epithelial cells derived from a colon cancer patient (HT-29) were used. They were processed exactly as the biopsies after fixation. Fluorescence histogram profiles were gated based on the negative control and analyses of cells that stained positively for the antibody were determined relative to this control.

**Measurement of γH2AX staining**

Fluorescence histogram profiles obtained from flow cytometric detection of the γH2AX staining were analyzed for the following two parameters: (a) relative percentage of cells that were positive, and (b) relative mean fluorescence intensity based on a region drawn according to the histogram profile of the negative control, expressed in light units. Rather than a single parameter (% positive cells), the average of these two parameters more accurately represents DNA damage because it not only indicates the percentage of cells with DNA damage but also the extent of damage within each positive cell (higher fluorescence means more damage).

We further standardized the degree of γH2AX staining by dividing the average of the two parameters obtained for each biopsy by the corresponding value obtained for the negative controls [14, 15]. Therefore, when the degree of staining of one biopsy was the same as that of the negative control, γH2AX staining was expressed as 1.

**Method assay validation**

1. HT-29 human colonic epithelial cells were seeded in plates with a diameter of 3.5 cm at a density of $5 \times 10^5$ cells/mL. Cell culture was maintained in DMEM/RPMI medium (50%/50% v/v) containing 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂.

2. Confluent cultures were treated with PBS (negative control), or 25 µM etoposide (positive control), for 1 hour in the dark at 37 °C in a 5% CO₂ tissue culture incubator.

3. Adherent cells were scraped from the plates, centrifuged then the pellet was resuspended in 2% paraformaldehyde/PBS for 1 hour at 4 °C.

4. Cells were centrifuged, the pellet resuspended in PBS, then centrifuged again.

5. The cell pellet was resuspended in 100 units/mL collagenase V in PBS solution for 2 hours at 37 °C with gentle rotation.

6. Cells were centrifuged and washed with PBS then incubated in 0.1% saponin/PBS for 1 hour at room temperature to permeabilize them prior to antibody incubation.

7. Antibody to γH2AX (0.5 µg/mL solution in 0.1% saponin/PBS) was added and cells were incubated for 1 additional hour at room temperature.

8. Antibody-stained cells were washed with PBS and then resuspended in 200µL of PBS/2% bovine serum before being run through a flow cytometer.

9. The γH2AX expression level was quantified by flow cytometry, analyzing $10^4$–$20000$ cells.

10. Fluorescence histogram profiles were analyzed for the mean fluorescence intensity (MFI) and % cells that were positive, based on a region drawn according to the histogram profile of the negative control (green open histogram in ▶ Fig.e1). In this example, the high MFI value of 95.39 shows that DNA of the colonic epithelial cells exposed to a genotoxic chemotherapeutic drug (red-filled histogram) shows a high reactivity to the fluorescent antibody to γH2AX compared with the MFI value of 11.15 for the negative control PBS-treated cells. To calculate the % positive cells, an arbitrary gate on the negative control is set to 10% positive staining. Relative to this control gate, 70% of etoposide-treated cells show damaged DNA.

**Results of assay validation**

The results of this analysis are summarized in ▶ Table e1. For the validation with 10 independent experiments, the negative control MFI & % gated values varied such that the slope of the histogram curve varied and the histogram shifted to the left and right of the x axis. This variation, which may be due to slight differences in cell culture, fixation, and staining conditions, was calculated as the coefficient of variation (CV) for both parameters. The mean and standard deviation (SD) of both parameters for the 10 experiments were calculated to yield the ratio: $CV = SD/mean = 0.5$ for the negative control. Therefore 1.5 was set as the threshold for the negative control. Consequently, samples with an average MFI and % gated cells higher than 1.5 were considered positive (i.e. suffering from potential DNA damage).
Analysis of γH2AX in biopsy specimens was performed concurrently with negative and positive controls. Analysis of γH2AX was blinded: the personnel of the analytical laboratory were not aware of the subject, the anatomical location, or to which study visit of γH2AX sample related. At the end of the study, the results were sent to the contract research organization (CRO) Biometry Unit responsible for the data evaluation after database lock. The unique biopsy identification numbers were reconciled with patients’ identifications using the investigator’s decoding list.

**Method for detection of DNA damage from biopsy specimens**

1. Biopsy samples received from a clinical study site were stored at 4 °C for up to 1 month.

2. Tissue samples were placed in individual 50-mL tubes containing 40 mL PBS and soaked for 1 hour at room temperature to rinse off residual fixative.

3. Tissues were transferred to 15-mL tubes containing 0.5 mL of 100 units/mL collagenase solution in PBS. The control cells were processed in parallel starting at this step, in separate tubes containing collagenase. Treatment was for 2 hours at 37 °C with gentle rotation.

4. Cells were centrifuged and washed with PBS then incubated in 0.1 % saponin/PBS for 1 hour at room temperature to permeabilize them prior to antibody incubation.

5. Antibody to γH2AX (0.5 µg/mL solution in 0.1 % saponin/PBS) was added and cells incubated for 1 additional hour at room temperature.

6. Antibody-stained cells were washed with PBS and then resuspended in 200 µL of PBS/2 % bovine serum before being run through a flow cytometer.

7. The γH2AX expression level was quantified by flow cytometry, analyzing 10 000–20 000 cells.

8. Fluorescence histogram profiles were analyzed for the MFI and % cells that were positive, based on a region drawn according to the histogram profile of the negative control.

### Table 1 Results of γH2AX assay validation.

<table>
<thead>
<tr>
<th>Parameter #1</th>
<th>Parameter #2</th>
<th>Parameter #1</th>
<th>Parameter #2</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>MFI</td>
<td>% gated</td>
<td>Relative MFI</td>
<td>Relative % gated</td>
</tr>
<tr>
<td>Negative</td>
<td>11.15</td>
<td>10.34</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Positive</td>
<td>95.39</td>
<td>70.05</td>
<td>8.56</td>
<td>6.77</td>
</tr>
</tbody>
</table>

MFI, mean fluorescence intensity.

**Appendix e3 Statistical analysis**

The inclusion of subjects in the full analysis set (FAS) and per protocol (PP) populations was performed prior to the statistical analysis. The safety analysis was performed on the safety set, whereas the efficacy analysis was performed on the FAS and PP sets.

Statistical analysis of the γH2AX biopsy staining was performed with the Prism Statistical Program using 2-way ANOVA with Holm–Sidak’s multiple comparisons test. This yielded P values of the negative control vs. patient biopsies and positive control vs. patient biopsies.

Although the planned sample size was not calculated by a statistical power analysis owing to lack of previous data, the reliability of the assay was evaluated based on guidelines that were formulated for testing the robustness of high-throughput screening assays [16]. This yields a value called the Z factor which gives an indication of the separation between the negative and positive hits (Z factor = S/R, where S [separation band] = negative mean + 3 × SEM, and R [dynamic range] = positive mean – 3 × SEM). An assay with a Z factor > 0.5 is considered to be excellent [16].

The average γH2AX staining for the 50 biopsies before and 50 biopsies after methylene blue administration were paired as a cumulative group to calculate the mean and 95 % confidence interval (CI). The statistical pairing is exact in that the region sampled from each patient before methylene blue was paired with the same region from the same patient after methylene blue. In addition, the histograms of each staining were tested for statistical differences by comparing five parameters: mean, median, geometric mean, standard deviation, and coefficient of variation. This additional unbiased analysis of the γH2AX staining before and after methylene blue administration is independent of the negative control because no gates were imposed on the histogram profiles. Paired 2-tailed t tests were performed using the Prism Program.
Fig. e4 Results of the γH2AX staining using the average of the two parameters obtained, with each parameter analyzed on 10 000 – 20 000 cells extracted from each biopsy specimen. Where values are shown as scatter plots, horizontal lines represent the means with the 95% confidence intervals (CIs) as error bars.

a Results for the 10 biopsy specimens from each of the 10 patients. For the controls, the 95% CIs are shown as solid bars, with the mean and standard error of the mean (SEM) shown inside the bars to indicate the separation band between the negative and positive controls and the dynamic range of the assay. The plotted values are also listed in the table below the graph. Neg Con, negative control; Pos Con, positive control. 

b Results for the paired biopsy specimens from the five different colonic sites sampled. The values before and after methylene blue administration are represented as filled and open symbols, respectively. The P values in each of the five regions and overall are listed in the table below the graph. C, cecum; Ac, ascending colon; Tc, transverse colon; Dc, descending colon; SR, sigmoid colon and rectum.