Thieme

Evaluation of genotoxicity related to oral methylene blue chromoendoscopy

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Appendix e1 – e3, Fig. e1, e4, Table e1
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ABSTRACT

Background An oral formulation of methylene blue with colonic delivery (MB-MMX) has been developed to increase detection of colorectal polys during colonoscopy. Traditionally, there have been safety concerns regarding DNA damage when methylene blue is exposed to white light. The aim of this study was to evaluate DNA damage in colonic mucosa after MB-MMX chromoendoscopy.

Methods This was an open-label phase II safety study to assess for genotoxicity on colorectal biopsies of patients undergoing two sequential colonoscopies before and after an oral dose of 200 mg MB-MMX added to their bowel prep. Analysis of a biomarker of double-stranded DNA breaks, γH2AX, was performed on biopsy specimens.

Results Ten patients were included in the study. The mean expression of γH2AX ± 95 % confidence intervals of the 50 biopsies before and after MB-MMX administration were 0.58 ± 0.08 and 0.62 ± 0.09, respectively (P = 0.24). None of the analyzed samples showed excess positive γH2AX expression, confirming the absence of DNA damage on biopsies after methylene blue exposure. No deaths or serious adverse events occurred.

Conclusions An oral dose of 200 mg of MB-MMX did not result in any detectable DNA damage.

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NCT02295774
TRIAL REGISTRATION: ■ Please provide us the full details Type of study (e. g. Single-Center, Randomized, prospective trial) ■ NCT02295774 at clinicaltrials.gov

Introduction

Pan-chromoendoscopy with methylene blue has been proven to increase the detection of neoplastic lesions in several settings, including Lynch syndrome, inflammatory bowel disease (IBD), and average-risk colonoscopy screening [1 – 4]. Both European and US guidelines recommend pan-chromoendoscopy for surveillance of high risk groups [5 – 8].

An oral formulation with colonic delivery of methylene blue (MB-MMX) has been developed to facilitate chromoendoscopy. However, safety concerns have been raised regarding the use of methylene blue, both in general [9] and in colonoscopy, as some studies have suggested that methylene blue in combination with white light can induce DNA damage [10, 11].

The aim of the present study was to investigate whether oral administration of methylene blue prior to colonoscopy had any effect on the colonic epithelial DNA, as assessed by in vitro anal-

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ysis with the most up-to-date and accurate biomarker of double-stranded DNA breaks.

Methods

Study design and population

This was a single-center, open-label, phase II study. The primary objective was an evaluation of the effect of 200 mg of oral methylene blue tablets on colonic epithelial DNA damage in comparison to standard white-light colonoscopy. Secondary end points were evaluation of the staining quality, safety, and tolerability of oral MB-MMX tablets.

Outpatients scheduled for colonoscopy who presented with pathological findings requiring a second colonoscopy were enrolled. The study inclusion/exclusion criteria are detailed in Appendix e1 (available online in Supplementary Material [12]). Biopsies were collected from each of five colonic regions: the cecum, the ascending, transverse, and descending colon, and the sigmoid/rectum. If a patient's inclusion criteria were confirmed, the patient was given their supply of MB-MMX tablets and their bowel cleansing preparation for their second colonoscopy.

Second colonoscopies were performed within 2 weeks. Biopsies were taken for γH2AX determination from normal-appearing mucosa that visually was the most stained in each of the five colonic regions.

Investigational treatments, dose regimen, and follow-up

The MB-MMX tablet is an oral colon-release formulation in the form of a coated tablet that contains methylene blue at a dose of 25 mg/tablet. The tablets are formulated using a multimatrix structure (MMX) that allows delivery in the colon and therefore higher staining intensity. All study participants took 4 L polyethylene glycol (PEG)-based bowel prep before colonoscopy and a total of eight MB-MMX tablets (200 mg in total).

Discharged patients were called within 2 weeks of their second colonoscopy to assess their health conditions and to record any adverse reactions.

Ethical procedures

The study was approved by an independent institutional review board (IRB) and performed in accordance with the guidelines and the Declaration of Helsinki. The study was registered on ClinicalTrial.gov (NCT02295774).

MB-MMX tablets were given free of charge by Cosmo Technologies Ltd., Ireland, with no other form of financial support being provided in the present study.

DNA damage assay using γH2AX biomarker

A form of histone 2A, denoted H2AX, is rapidly phosphorylated (γH2AX) in response to DNA double-strand damage and is considered an excellent biomarker of DNA damage [13–15]. Details are reported in Appendix e2 (▶ **Fig. e1**, ▶ **Table e1**; available online in Supplementary Material).

Statistical analysis

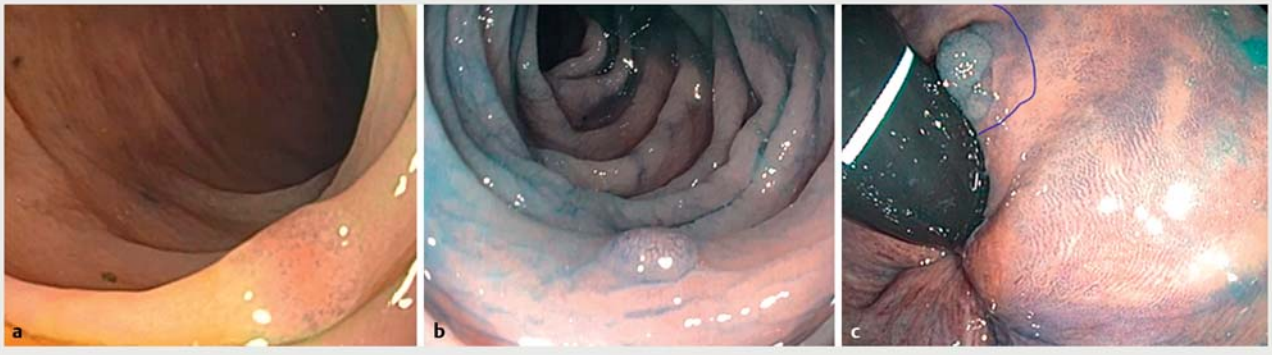
The statistical analysis is described in Appendix e3 (available online in Supplementary Material; [16]).

Results

Ten patients (7 men, 3 women; mean age 63.1 years) were included in the study. The staining quality of the colonic mucosa was acceptable (at least 50 % of colonic mucosa stained) in the ascending, transverse, and descending colon, while it was lower (detectable) in the rectosigmoid (▶ **Fig. 2**).

None of the analyzed samples showed excess positive γH2AX staining relative to the negative control (n = 10; safety set) for colonoscopies performed both with and without methylene blue (MB-MMX).

When all 50 biopsies from the before and after methylene blue colonoscopies were grouped and compared (▶ **Fig. 3**), the normalized γH2AX staining values were 0.58 ± 0.08 and 0.62 ± 0.09 (mean ± 95 % confidence interval), respectively (P = 0.24) Comparison of the paired results for individual subjects, before and after methylene blue administration, from each of the five colonic regions sampled showed an overlap between the stain-



▶ **Fig. 2** Endoscopic views after the administration of methylene blue (MB-MMX) showing sessile polyps in: **a** the ascending colon; **b** the transverse colon; **c** the rectum (retroflexed view).

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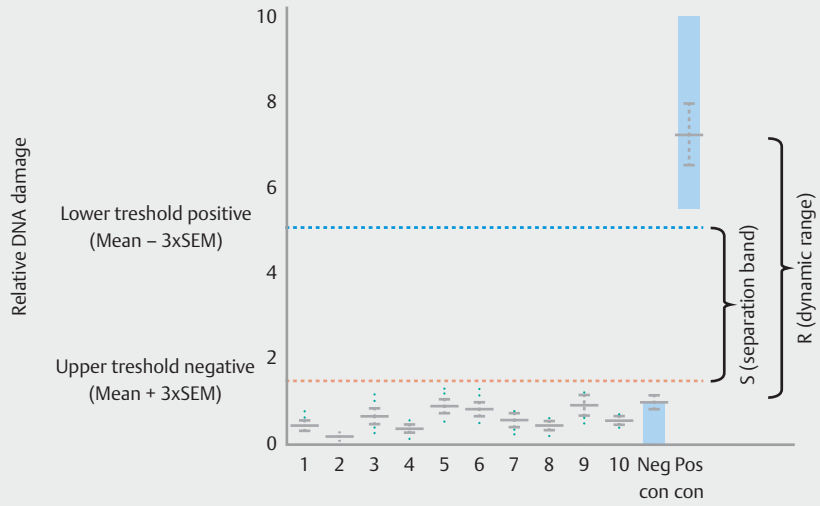

a

Patient or control	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	Neg con	Pos con
Mean of 10 biopsies	0.460	0.203	0.674	0.380	0.913	0.844	0.583	0.452	0.928	0.576	1.000	7.225
Std. error of mean	0.049	0.017	0.082	0.037	0.072	0.082	0.066	0.047	0.114	0.038	0.163	0.728
Lower 95 % CI of mean	0.349	0.165	0.489	0.295	0.750	0.658	0.434	0.345	0.670	0.489	0.631	5.445
Upper 95 % CI of mean	0.571	0.241	0.860	0.465	1.076	1.030	0.732	0.559	1.186	0.663	1.369	9.005

▶ **Fig. 3** Comparison for all 10 patients before and after methylene blue (MB-MMX; 50 specimens before vs. 50 specimens after). **a** Scatter plots showing the average value of the two parameters obtained from the γH2AX staining of the biopsies from all 10 patients. The horizontal line through the data points indicates the mean of the points and the error bars represent the 95 % confidence interval (CI). The mean ± 95 %CIs are 0.58 ± 0.08 and 0.62 ± 0.09 for the before and after sets, respectively ($P = 0.24$).

(Continuation see following page)

ing histograms ($P > 0.05$ for all) (▶ **Fig. e 4a** , available online in Supplementary Material). Therefore, there was no statistically significant difference between the before and after methylene blue administration biopsies.

Statistical analysis of the sample set against the positive and negative controls indicates a robust screening assay when the upper threshold of the negative range is strictly defined as the $\text{mean}_{(\text{negative control})} + 3 \times$ standard error of the mean (SEM) and the lower threshold of the positive range is defined as the $\text{mean}_{(\text{positive control})} - 3 \times \text{SEM}$ (shown by the dashed lines in ▶ **Fig. e 4b**). The calculated Z factor of 0.65 is indicative of a well-separated negative and positive range. More importantly, highly significant P values were obtained for the $\text{mean}_{(\text{positive control})}$ vs. the $\text{means}_{(\text{biopsy sample})}$ ($P < 0.001$ for all samples). While the same analysis comparing the $\text{mean}_{(\text{negative control})}$ vs. the $\text{means}_{(\text{biopsy sample})}$ indicate that all 10 samples from all 10 patients are negative for γH2AX (negative control vs. patients not significantly different, $P > 0.99$ for all). Therefore, we can conclude with a high degree of statistical confidence that all of the samples analyzed were negative for γH2AX staining.

Secondary safety evaluation

No deaths or serious adverse events (SAEs) occurred. Overall, 13 mild treatment-emergent adverse events (TEAEs) occurred, as reported in ▶ **Table 2**.

Discussion

According to our study, the oral administration of 200 mg MB-MMX did not affect the colonic epithelial double-stranded DNA. The lack of DNA damage was consistent across all of the 10 patients and in all of the 50 biopsies that were taken across the five colonic regions. These results are relevant primarily because MB-MMX represents a promising approach to minimize loss of time in pan-chromoendoscopy that, in turn, has been shown to be effective in both the surveillance of high risk groups and in average-risk colorectal cancer screening [1 – 8]. However, there have been concerns about the genotoxic potential of methylene blue [9, 17].

The results of our study suggest such risk is probably negligible. The discrepancy with previous studies may be due to the

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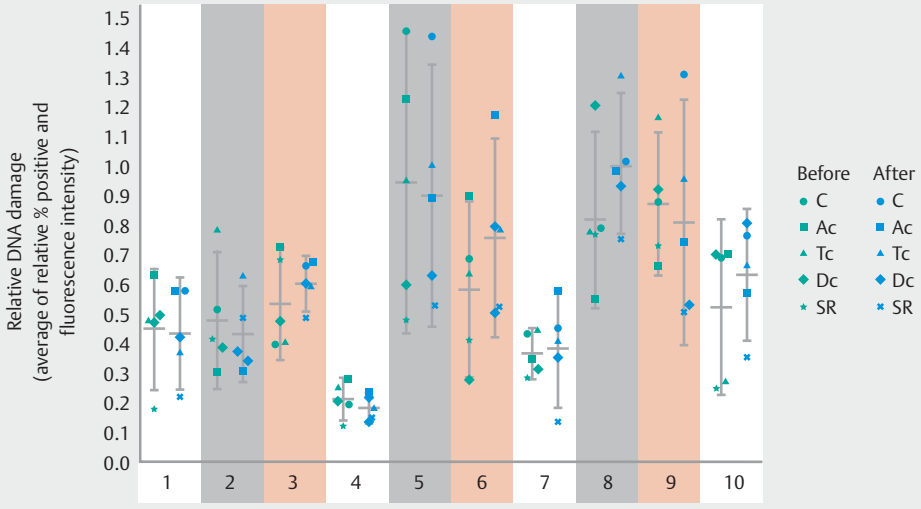

b

	Colon region	Caecum	p value	Ascending	p value	Transverse	p value	Descending	p value	Sigma/rectum	p value	AVE of 5 regions	Overall p value before vs after
Patient #1	Before	0.48	0.11	0.64	0.43	0.49	0.63	0.50	0.49	0.19	0.51	0.46	0.68
	After	0.59		0.59		0.38		0.43		0.23		0.44	
Patient #2	Before	0.52	0.53	0.31	0.34	0.79	0.78	0.39	0.46	0.42	0.10	0.49	0.32
	After	0.35		0.31		0.64		0.38		0.49		0.43	
Patient #3	Before	0.40	0.35	0.73	0.14	0.41	0.46	0.45	0.42	0.69	0.40	0.54	0.47
	After	0.67		0.68		0.60		0.61		0.49		0.61	
Patient #4	Before	0.20	0.36	0.29	0.78	0.26	0.63	0.21	0.59	0.13	0.32	0.22	0.22
	After	0.22		0.24		0.19		0.14		0.15		0.19	
Patient #5	Before	1.46	0.52	1.23	0.26	0.96	0.59	0.61	0.99	0.49	0.56	0.95	0.57
	After	1.44		0.90		1.01		0.64		0.54		0.90	
Patient #6	Before	0.69	0.36	0.90	0.34	0.64	0.40	0.32	0.32	0.42	0.39	0.59	0.20
	After	0.51		1.18		0.79		0.80		0.53		0.76	
Patient #7	Before	0.44	0.35	0.35	0.51	0.45	0.35	0.32	0.43	0.29	0.31	0.37	0.78
	After	0.46		0.58		0.41		0.36		0.14		0.39	
Patient #8	Before	0.80	0.36	0.56	0.32	0.78	0.36	1.21	0.36	0.77	0.34	0.82	0.29
	After	1.02		0.99		1.31		0.94		0.76		1.00	
Patient #9	Before	0.88	0.35	0.67	0.34	1.17	0.38	0.93	0.36	0.73	0.38	0.88	0.69
	After	1.31		0.75		0.96		0.53		0.51		0.81	
Patient #10	Before	0.69	0.42	0.71	0.25	0.28	0.21	0.71	0.63	0.25	0.63	0.53	0.26
	After	0.77		0.58		0.67		0.81		0.36		0.64	

▶ **Fig. 3** (Continuation) **b** Scatter plot of the mean differences between the paired biopsies.

relatively high doses over long periods that were previously used, resulting in significantly higher exposure to methylene blue than would result from a single 200-mg MB-MMX dose at the expected frequency of a routine colonoscopy [18]. One study has suggested methylene blue could induce colonic DNA damage using an in vitro simulation of chromoendoscopy on biopsies taken from colonic mucosa after exposure to 0.1 % methylene blue spraying [10]. The study showed that, among 10 patients exposed to methylene blue, eight had higher levels of DNA damage, as measured by alkaline comet assay, and all had higher levels of DNA damage, measured by the FPG-modified comet assay.

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▶ **Table 2** Number of reported treatment-emergent adverse events (TEAEs) described by system organ class (SOC) and preferred term (PT), and number of subjects reporting these TEAEs.

MedDRA description (SOC term)	MedDRA description (PT term)	Methylene blue MMX tablets	
		Number of TEAEs	Number of subjects
Renal and urinary disorders		8	8 (80.0 %)
	Chromaturia	8	8 (80.0 %)
Gastrointestinal disorders		4	4 (40 %)
	Feces discolored	4	4 (40 %)
Nervous system disorders		1	1 (10 %)
	Headache	1	1 (10 %)

MedDRA, Medical dictionary for regulatory activities.

In contrast our results, from a similar group of 10 consecutive patients, led to opposite conclusions based on a very up-to-date and specific test to identify DNA damage, with a methodology that not only indicates the percentage of cells with DNA damage but also the extent of damage within each positive cell. We believe that the limitation of the small group size is overcome by the rigorous methodology used for the present study. In contrast to previous qualitative methods to detect DNA damage, detection of γH2AX has been shown to be considerably more sensitive, efficient, and reproducible [13, 14, 19]. Statistical analyses indicate a robust screening assay with highly significant *P* values.

Second, in our study white-light exposure time was longer in patients exposed to MB-MMX (22.8 vs. 17.5 minutes) because they all required polyp resection. This has created the worst conditions in which to evaluate DNA damage as it has been postulated that the risk of genotoxicity increases with time of exposure to white light. Moreover, this condition consistently reproduces the typical clinical setting where, after methylene blue chromoendoscopy, detection of flat lesions may require extended operative time and light exposure. Third, we excluded bias related to inter-individual variability by collecting biopsies from mucosa in each colonic region twice in the same patient – without and with methylene blue staining – with a 15-day interval. We found no statistically significant differences between these paired samples. Moreover, the second colonoscopy performed within 2 weeks of the first did not affect the DNA analysis, because of the short time interval between the two.

The use of tablets recreates similar conditions for pan-chromoendoscopy, with the advantage of increasing the intestinal lesion detection rate and therefore is ideal, especially in high risk patients, such as those affected by IBD. Pan-chromoendoscopy requires 60 – 100 mL of 0.1 % methylene blue or 60 – 100 mg of methylene blue to spray the entire colorectal mucosa [20]. Despite the dose of methylene blue in the tablets being higher (200 mg), the latter are time-saving and much simpler to use. An additional use of methylene blue is to define the borders of any focal lesions. On the one hand, this approach requires a smaller amount of methylene blue; on the other, it lacks the possibility of increasing the lesion detection rate.

Because of its exploratory nature, our study does however have some limitations, namely small sample size, light exposure limited to a maximum of 23 minutes, and an unvalidated scale for the detection of methylene blue staining. With regard to the last limitation, our aim was to assess only qualitatively whether MB-MMX was associated with DNA damage.

In conclusion, based on these preliminary results, we can affirm that an oral dose of 200 mg MB-MMX seems to be safe and that the increase in DNA lesions reported by others upon methylene blue – light treatment is unlikely to have in vivo biologic significance.

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Competing interests

None.

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In brief:

Proof-of-concept study showing that methylene blue administered orally did not result in any detectable DNA damage in colonic mucosal cells.

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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 childbearing 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.

Appendix e2 Analytics for γH2AX assay

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.

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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 5×10^5 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 % CO_2.

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 % CO_2 tissue culture incubator.

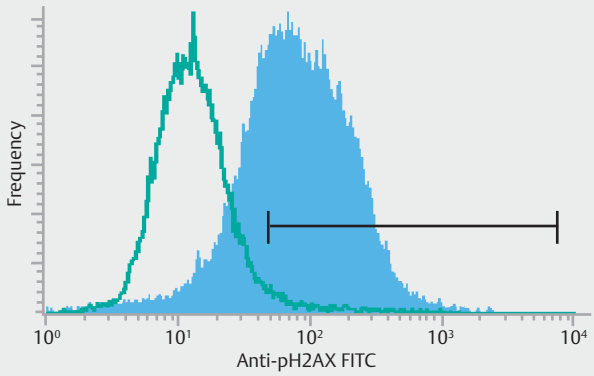
3 Adherent cells were scraped from the plates, centrifuged then the pellet was resuspended in 2 % paraformaldehyde/PBS for 1 hour at 4 °C.

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.



▶ **Fig. e1** Histogram profile for γH2AX expression quantified by flow cytometry. The green open histogram represents the negative control.

8 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.

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

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▶ **Table e1** Results of γH2AX assay validation.

	Parameter #1	Parameter #2	Parameter #1	Parameter #2	Both parameters
Description	MFI	% gated	Relative MFI	Relative % gated	Average result
Negative	11.15	10.34	1.00	1.00	1.00
Positive	95.39	70.05	8.56	6.77	7.66

MFI, mean fluorescence intensity.

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.

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.

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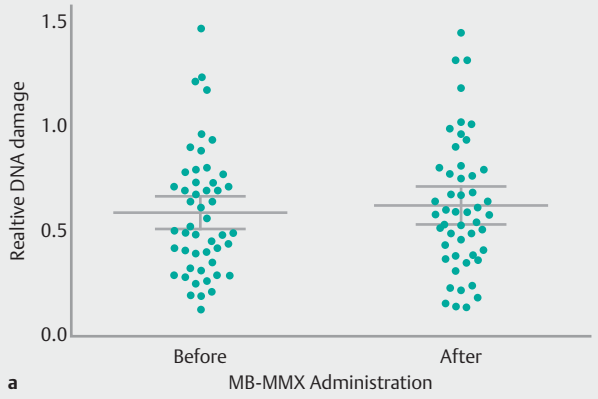

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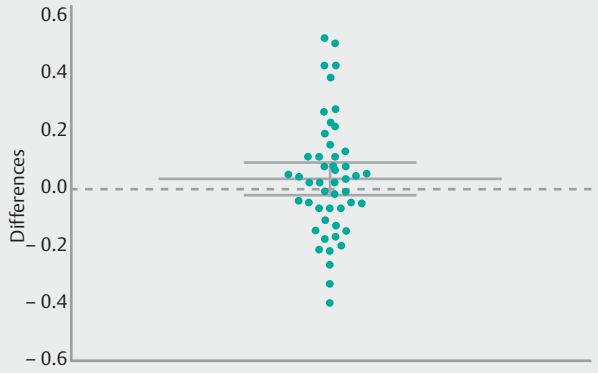

b

▶ **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.

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