DNA repair by MGMT, but not AAG, causes a threshold in alkylation-induced colorectal carcinogenesis

Jörg Fahrer*, Janina Frisch, Georg Nagel, Alexander Kraus, Bastian Dörsam, Adam D. Thomas, Sonja Reißig¹, Ari Waisman¹ and Bernd Kaina

Institute of Toxicology and ¹Institute of Molecular Medicine, University Medical Center Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

*To whom correspondence should be addressed. Tel: +49 6131 179260; Fax: +49 6131 178499; Email: fahrer@uni-mainz.de

Correspondence may also be addressed to Bernd Kaina. Tel: +49 6131 179217; Fax: +49 6131 178499; Email: kaina@uni-mainz.de

Abstract

Epidemiological studies indicate that N-nitroso compounds (NOC) are causally linked to colorectal cancer (CRC). NOC induce DNA alkylations, including O⁶-methylguanine (O⁶-MeG) and N-methylated purines, which are repaired by O⁶-MeG-DNA methyltransferase (MGMT) and N-alkyladenine-DNA glycosylase (AAG)-initiated base excision repair, respectively. In view of recent evidence of nonlinear mutagenicity for NOC-like compounds, the question arises as to the existence of threshold doses in CRC formation. Here, we set out to determine the impact of DNA repair on the dose–response of alkylation-induced CRC. DNA repair proficient (WT) and deficient (Mgmt⁻/⁻, Aag⁻/⁻ and Mgmt⁻/⁻/Aag⁻/⁻) mice were treated with azoxymethane (AOM) and dextran sodium sulfate to trigger CRC. Tumors were quantified by non-invasive mini-endoscopy. A non-linear increase in CRC formation was observed in WT and Aag⁻/⁻ mice. In contrast, a linear dose-dependent increase in tumor frequency was found in Mgmt⁻/⁻ and Mgmt⁻/⁻/Aag⁻/⁻ mice. The data were corroborated by hockey stick modeling, yielding similar carcinogenic thresholds for WT and Aag⁻/⁻ and no threshold for MGMT lacking mice. O⁶-MeG levels and depletion of MGMT correlated well with the observed dose–response in CRC formation. AOM induced dose-dependently DNA double-strand breaks in colon crypts including Lgr5-positive colon stem cells, which coincided with ATR-Chk1-p53 signaling. Intriguingly, Mgmt⁻/⁻ mice displayed significantly enhanced levels of γ-H2AX, suggesting the usefulness of γ-H2AX as an early genotoxicity marker in the colorectum. This study demonstrates for the first time a non-linear dose–response for alkylation-induced colorectal carcinogenesis and reveals DNA repair by MGMT, but not AAG, as a key node in determining a carcinogenic threshold.

Introduction

Colorectal cancer (CRC) is the third most common cancer type, affecting more than one million people worldwide (1). A number of risk factors have been linked to CRC etiology. Among those, red and processed meat intake was shown to significantly increase the risk for CRC formation (2) and, intriguingly, correlates with a higher mortality rate in patients with non-metastasized disease (3). Red meat contains high levels of heme iron that promotes the formation of N-nitroso compounds (NOC) in the large bowel, a process that is thought to play a fundamental role in the etiology of sporadic CRC (4). NOC, including N-nitrosamines, which are found in food, beverages, tobacco smoke and cosmetics, are S⁰¹ alkylating agents that react with DNA following metabolic activation by cytochrome P450 2E1 (CYP2E1). NOC induce a plethora of DNA lesions (5), which primarily comprise the N-methylated DNA bases N3-methyladenine (N3-MeA) and N7-methylguanine (N7-MeG) as well as O⁶-methylguanine (O⁶-MeG). The latter is repaired by the suicide enzyme O⁶-methylguanine-DNA methyltransferase (MGMT) in a one-step reaction, leading to inactivation and proteasomal degradation of MGMT (6). The N-methylated DNA bases N3-MeA and N7-MeG are substrates of the base excision repair (BER) pathway, which is primed by N-alkyladenine-DNA glycosylase (AAG). The removal
of the damaged base by AAG generates an apurinic site, which is subsequently processed by other BER components to restore DNA integrity (7). Thus, MGMT and AAG-initiated BER are the major DNA repair pathways that protect against the detrimental and carcinogenic effects induced by NOC (4,7).

There is substantial evidence that MGMT represents an important barrier against NOC-induced colorectal carcinogenesis. Genetic abrogation of Mgmt in rodents results in a markedly enhanced number of aberrant crypt foci and colorectal tumors induced by the colonotropic agent N-methyl-N-nitrosourea (AOM) (8). Furthermore, Mgmt knockout animals were shown to be highly susceptible to AOM-induced CRC formation in a setting of chronic inflammation triggered by dextran sodium sulfate (DSS) (9). Consistent with these studies, Mgmt promoter hypermethylation has been linked to the etiology of sporadic human CRC found in ~40% of sporadic colorectal tumors (10). This methylation appears to be an early event in colorectal carcinogenesis and is also positively correlated with tumor progression (10,11). Epigenetic inactivation of Mgmt is strongly associated with the presence of guanine to adenine mutations in the Kras proto-oncogene as a transforming event (12,13). In contrast to MGMT, little is known about the role of AAG in sporadic CRC formation. Studies with transgenic Aag−/− animals displayed a higher number of alkylation-induced colorectal tumors as compared to wild-type (WT) mice (9) and the formation of dysplastic adenomas following severe chronic inflammation even in the absence of AOM (14).

A growing body of evidence suggests that DNA repair is a crucial determinant in the dose-response of alkylation agent-induced mutagenicity. A study performed in lymphoblastoid cells demonstrated that S2,2 alkylation agents, such as ethylmethane sulfonate, display non-linear dose-responses for mutagenicity (15), allowing for the calculation of a point of departure (16). This was subsequently confirmed in animal experiments, showing lack of mutagenicity at low dose levels (17). Recent work provided further evidence for the existence of a ‘no observed genotoxic effect level’ after treatment with the NOC-like alkylation agent N-methyl-N-nitrosourea and showed that inactivation of MGMT critically impacts the tolerance to N-methyl-N-nitrosourea with a reduced no observed genotoxic effect level in lymphoblastoid cells (18). Carcinogenesis is clearly much more complex than mutagenicity and genotoxicity as a measurable endpoint. Consequently, there are no studies available addressing the existence of a point of departure in alkylation-induced CRC formation.

In view of the essential role of DNA repair as a barrier against NOC-induced mutations and CRC, we determined the impact of the main alkylation repair pathways, MGMT and AAG, on the dose-response of NOC-induced CRC. We also set out to determine whether CRC formation correlates with the early DNA damage induction and alkylation-triggered DNA damage response (DDR) in colorectal tissue. The study demonstrates that DNA repair by MGMT, but not AAG, is responsible for a threshold in alkylation-induced CRC, although both MGMT and AAG protect against NOC-induced colorectal cancer.

### Materials and methods

#### Mouse models, induction of colorectal carcinogenesis and mini-endoscopy

Mgmt-null, Aag-null and Mgmt/Aag-double null (DKO) mice in a C57BL/6 background were described previously (9). Eight to 14-week-old sex-matched Mgmt, Aag, DKO and C57BL/6 WT control mice were used. All animal experiments were approved by the government of Rhineland-Palatinate and the Animal Care and Use Committee of the University Medical Center Mainz and performed in agreement with the German federal law and the guidelines for the protection of animals. The AOM/DSS model was used to induce alkylation damage-initiated, colitis-associated CRC (Figure 1A). AOM was dissolved in water and diluted in phosphate-buffered saline (PBS) [0–10 mg/kg body weight (BW); Sigma–Aldrich, Deisenhofen, Germany]. It was administered as a single intraperitoneal injection followed by two cycles of 1% DSS (MP Biomedicals, Ilkirch, France) in drinking water. CRC development was analyzed 16 weeks after AOM injection using a high-resolution mini-endoscopy unit (Karl Storz, Tuttingen, Germany). Anaesthetized mice were subjected to mini-endoscopy (19) and tumors were scored as to their number and size for each treatment group (Figure 1B).

#### Tissue collection

To analyze the alkylation-induced DDR, animals were treated with AOM and killed after 24 and 48 h. Colon and liver tissues were carefully dissected, flushed with PBS and fixed in neutral buffered formaldehyde solution (Roti®-Histofix; Carl Roth, Karlsruhe, Germany) or snap-frozen in liquid nitrogen.

#### Immunohistochemistry and confocal microscopy

Fixed colorectal tissue was embedded in paraffin, sectioned at 5 μm and processed for immunohistochemistry (9,20). Staining of γ-H2AX, proliferating cell nuclear antigen (PCNA), Lgr5, O6-MeG and in situ labeling of apoptotic cells was performed as follows. Briefly, sections were deparaffinized and rehydrated followed by antigen retrieval. Non-specific binding sites were blocked with protein blocking solution (DAKO, Hamburg, Germany) and sections were incubated with a γ-H2AX antibody (1:400; Abcam, Cambridge, UK) overnight at 4°C. After incubation with an Alexa488-coupled secondary antibody (1:600; Life Technologies, Darmstadt, Germany) for 2 h at room temperature (RT), DNA was counterstained with TO-PRO-3 dye (1:100; Life Technologies, Darmstadt, Germany) for 30 min at RT. Slides were mounted with Vectashield medium (Linaris, Dossenheim, Germany) and analyzed by confocal microscopy with a Zeiss Axio Observer.Z1 microscope equipped with a LSM710 laser-scanning unit (Zeiss, Oberkochen, Germany). Images were acquired in optical sections of 1 μm and processed with ImageJ version 1.45 (NIH).

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. Colorectal tissue sections were stained with the fluorescein in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions and analyzed by confocal microscopy. For γ-H2AX/TUNEL double staining, sections were first subjected to TUNEL assay followed by γ-H2AX immunostaining as described above, except that a Cy3-coupled secondary antibody (1:600; Jackson ImmunoResearch Laboratories, Suffolk, UK) was used.
MGMT activity assay

MGMT activity in colorectal and liver tissue was determined using a radio-active assay based on the transfer of $^3$H-labeled methyl groups from the O\textsuperscript{6}-position of guanine in calf thymus DNA to the MGMT protein. The assay was performed as previously reported \(23\). Extracts of MGMT-deficient HeLa MR cells served as negative control and HeLa S3 cell extracts as positive control. Two hundred µg of protein extract obtained from liver and colorectal tissue were used for the assay. Radioactivity of the samples was measured by scintillation counting and data were analyzed using GraphPad Prism 5.0 software.

DNA extraction and detection of O\textsuperscript{6}-MeG

O\textsuperscript{6}-MeG DNA adduct levels were determined as previously reported \(24\). Colon or liver tissue was homogenized and subjected to RNase A digestion followed by proteinase K incubation overnight. Genomic DNA was isolated by phenol–chloroform extraction, precipitated by 70% ice-cold ethanol and finally dissolved in tris-ethylidithylamine tetracetic acid (TE) buffer at pH 7.4. DNA concentration and purity were determined using a NanoDrop 2000 (Thermo Scientific, Dreieich, Germany). O\textsuperscript{6}-MeG levels were determined using an immuno spot slot assay. Firstly, DNA was heat-denatured followed by the addition of 2 M ammonium acetate. Thereafter, 500 ng of DNA were immediately vacuum-aspirated onto a positively charged nylon membrane (GE Healthcare, Munich, Germany) using a slot blot manifold. The membrane was then fixed for 90 min at 100°C and blocked with 5% (w/v) non-fat dry milk in PBS-T. O\textsuperscript{6}-MeG was detected by a monoclonal O\textsuperscript{6}-MeG antibody (Axoxxa, Farmingdale, USA) followed by a secondary peroxidase-coupled antibody (Santa Cruz Biotechnology, Germany) and enhanced chemiluminescence detection. Densitometric evaluation of blots was performed by Adobe® Photoshop® CS5 software and analyzed by GraphPad Prism 5.0 software.

Dose–response modeling

Analysis was performed according to the guidelines described recently \(25\,26\). Regression analysis was performed using SPSS version 21 for Windows (IBM, New York, NY). To determine the shape of the dose–response curves, the sum of squares for linear and quadratic fits was compared using an F-test kindly provided by Professor David Skibinski (Swansea University, UK). Quadratic curves (F-test alpha values of less than 0.05) were subjected to repeat regression analysis using doses below, and including, the no observed effect level (NOEL), as determined by Doak et al. (15) using SPSS version 21 for Windows. A flat slope below the NOEL, i.e. one with a coefficient of determination ($R^2$) that passed 0 (with standard deviations), were analyzed to determine the threshold dose (td) using the hockey stick model \(27\) in R for Mac version 3.0.0 (Vienna, Austria). Threshold doses could not be determined for dose–responses that did not satisfy the aforementioned criteria (i.e. linear dose–responses), therefore, NOELs and lowest observed effect levels were determined for all dose–responses and used as parameters to compare the DNA repair phenotypes.

Results

Lack of MGMT critically impacts the dose–response of NOC-induced CRC

To analyze the influence of DNA repair on the dose–response of NOC-induced CRC, we used DNA repair-proficient (WT) and -deficient mice (Mgmt\textsuperscript{−/−}, Aag\textsuperscript{−/−} and Mgmt\textsuperscript{−/−}Aag\textsuperscript{−/−} double knockout, DKO), which were subjected to increasing doses of AOM as part of the well-established AOM/DSS model of CRC (Figure 1A). Mini-endoscopy \(39\) was used to determine the colorectal tumor number and to perform tumor size grading (T1–T5, Figure 1B). Animals were challenged with increasing doses of AOM (0–7.5 mg/kg BW) followed by two cycles of DSS. Colonoscopy revealed a statistically significant increase in tumor number.

Figure 1. Assessment of colorectal tumors by mini-endoscopy. (A) AOM/DSS model of colorectal carcinogenesis. The colonotropic tumor initiator AOM is injected intraperitoneally (i.p.) into DNA repair-proficient and -deficient mice. Subsequently, the mice receive two cycles of 1% DSS in the drinking water, which triggers chronic colitis and tumor promotion. Tumor formation is monitored by non-invasive mini-endoscopy after 16 weeks. (B) Tumor size grading is performed in relation to the colonic circumference (T1 up to T5).

Preparation of tissue extracts and western blot analysis

Tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer and lysed by sonication on ice. Lysates were cleared by centrifugation and stored at -80°C. Protein content was determined by Bradford assay. Western blot analysis was performed as previously reported \(15\). Lysates were cleared by centrifugation and 30 µg of protein were loaded per lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a nitrocellulose membrane (Perkin Elmer, Rodgau, Germany) using a wet blot chamber (Bio-Rad, München, Germany). The membrane was blocked in 5% (w/v) non-fat dry milk in PBS-containing Tween-20 [0.1% (v/v), PBS-T] for 1 h and then probed with respective primary antibodies. After several washing steps with PBS-T, the membrane was incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). After the final washing steps, proteins were detected by chemiluminescence using Western Lightning® Plus-ECL (Perkin Elmer, Rodgau, Germany). Densitometric evaluation of blots was performed using Adobe® Photoshop® CS5 software and analyzed by GraphPad Prism 5.0 software (GraphPad software, San Diego, CA). The following primary antibodies validated previously \(22\) were used: antiheat shock protein (Hsp90) alfa (Santa Cruz, Heidelberg, Germany), anti-γ-H2AX (Abcam, Cambridge, UK), anti-p33, anti-phospho-Chk1, anti-phospho-Chk2 and anti-phospho ATR (all from Cell Signaling Technology, Denver, UK).
Interestingly, the tumor number
were not formed above the background level (Figure 2B and Supplementary Figure 1B, available at Carcinogenesis Online).

Interestingly, the tumor number in Aag−/− mice (Figure 2B) was almost twice as high as in WT animals at doses of 5 and 7.5 mg/kg BW (Figure 2E). In Mgmt−/− mice tumors were formed even at the low dose levels of AOM and increased in a dose-dependent manner (Figure 2C and Supplementary Figure 1C, available at Carcinogenesis Online), which was comparable to DKO mice (Figure 2D and Supplementary Figure 1D, available at Carcinogenesis Online). Please note that for MGMT-deficient animals treated with 7.5 mg/kg BW data are missing due to the high lethality of Mgmt−/− and DKO animals at this dose level. The dose–response of CRC formation for all strains is compiled in Figure 1E and F.

Statistical analysis of the data using the hockey stick model (27) revealed a non-linear dose–response relationship for WT and Aag−/− mice with similar carcinogenic td at 3.5 and 2.9 mg/kg BW, respectively, for tumor number (Supplementary Figure 2A and B, available at Carcinogenesis Online; Table 1) and 3.9 and 3.1 mg/kg BW, respectively, for tumor score (Supplementary Figure 2E and F and Table 1, available at Carcinogenesis Online). In contrast, Mgmt−/− and DKO animals displayed linear dose–responses for CRC formation (Supplementary Figure 2C, D, G and H, available at Carcinogenesis Online), without evidence of a carcinogenic threshold. Taken together, our data demonstrate a non-linear dose–response in NOC-induced CRC with a carcinogenic threshold and highlight the pivotal role of MGMT, but not AAG-initiated BER, as a barrier against AOM-induced cancer development at low doses. Of note, at high dose levels Aag−/− mice showed a higher response than the WT, supporting the notion that AAG is involved in cancer protection.

**Formation of O6-MeG adducts and MGMT activity in NOC-induced CRC**

We next studied whether the linear dose–response for CRC formation in Mgmt−/− and DKO mice was reflected on DNA adduct and MGMT activity level. Induction of O6-MeG over the dose range of AOM was assessed after 24 h in liver tissue, which represents the first target organ for AOM due to its metabolic activation. Confocal microscopy revealed a dose-dependent increase of O6-MeG adducts in Mgmt−/− mice with strong staining at 10 mg/kg BW (Figure 3A and Supplementary Figure 3B, available at Carcinogenesis Online), while O6-MeG staining was much weaker in AOM-treated WT mice and was not detectable below 5 mg/kg BW (Figure 3A and Supplementary Figure 3A, available at Carcinogenesis Online). Interestingly, O6-MeG DNA adducts partially colocalized in hepatocytes with phosphorylated histone 2AX (γ-H2AX), a well-established marker of DNA double-strand breaks (DSBs) (Figure 3D). To measure the O6-MeG levels quantitatively, genomic liver DNA was isolated 24 h after AOM treatment and subjected to slot blot analysis. In WT and Aag−/− animals, a borderline increase in O6-MeG levels was observed at 3 and 5 mg/kg BW (Figure 3B and C), while a high dose of 10 mg/kg

![Figure 2. Dose-dependent colorectal tumor formation in DNA repair-proficient and -deficient mice.](https://academic.oup.com/carcin/article-abstract/36/10/1235/316766)
Table 1. Modeling of dose–response curves (tumor number)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dose–response</th>
<th>NOEL/lowest observed effect level (mg/kg bw)</th>
<th>Threshold dose (mg/kg bw)</th>
<th>Lower CI (mg/kg bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Non-linear (P = 0.045)</td>
<td>5/7.5</td>
<td>3.45</td>
<td>1.12</td>
</tr>
<tr>
<td>Aag&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Non-linear (P &lt; 0.001)</td>
<td>3/5</td>
<td>2.88</td>
<td>2.00</td>
</tr>
<tr>
<td>Mgmt&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Linear (P = 0.999)</td>
<td>1/3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DKO</td>
<td>Linear (P = 1)</td>
<td>1/3</td>
<td>—</td>
<td>—</td>
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Figure 3. Assessment of O<sub>6</sub>-MeG levels and MGMT activity following AOM treatment. (A) Dose-dependent O<sub>6</sub>-MeG formation in liver tissue of WT and Mgmt<sup>−/−</sup> mice challenged with increasing doses of AOM as indicated. Representative images are shown. O<sub>6</sub>-MeG is shown in green, while nuclei are stained in blue. (B) Detection of O<sub>6</sub>-MeG in genomic liver DNA of WT and DNA repair-defective animals treated with increasing doses of AOM (0–10 mg/kg BW; n = 3 per dose and genotype). (C) Quantitative evaluation of the slot blots shown in (B). O<sub>6</sub>-MeG levels were calculated in relation to PBS-treated control mice (0 mg AOM/kg BW) and are presented as mean ± SEM. Statistical analysis was performed using unpaired, two-tailed Student’s t-test. ***P < 0.001, **P < 0.01, *P < 0.05, n.s. not significant. (D) Costaining of O<sub>6</sub>-MeG (green) and γ-H2AX (red) in liver tissue from Mgmt<sup>−/−</sup> animals 24 h after treatment with 10 mg AOM/kg BW. (E) MGMT activity in liver and colon of AOM-treated WT mice. Animals were treated with increasing doses of AOM (0–10 mg/kg BW; n = 3 per dose). After 24 or 48 h, MGMT activity was determined. Data are given as mean ± SEM.
BW led to significantly elevated adduct levels. It is important to note that at 48 h following low dose AOM treatment (3 mg/kg BW), O6-MeG levels in WT and Aag−/− mice approximated those of control animals (Supplementary Figure 4A and B, available at Carcinogenesis Online), reflecting the repair of adducts by MGMT over the 48 h post-exposure period. Intriguingly, Mgmt−/− and DKO animals displayed a linear AOM-induced formation of O6-MeG DNA adducts over the whole dose range tested (Figure 3B and C), which did not change during an additional 24 h post-exposure period (Supplementary Figure 4A and B, available at Carcinogenesis Online).

We also determined the MGMT activity in liver and colorectal tissue extracts of WT animals challenged with AOM. In the liver, AOM induced a dose-dependent depletion of MGMT activity 24 h after treatment. The MGMT activity was partially restored at low AOM doses after 48 h, but remained undetectable at the highest AOM dose used (Figure 3E). In colorectal tissue, treatment with AOM provoked a time- and dose-dependent reduction in MGMT activity after 24 h, with complete loss of activity at a dose of 10 mg/kg BW AOM after 48 h (Figure 3E). The observed delay in the decrease of MGMT activity in the colon is probably attributable to AOM metabolism, which is first activated in the liver and subsequently in the colon (19). In summary, our findings indicate a rather non-linear formation of O6-MeG DNA adducts in WT animals, which was accompanied by a depletion of MGMT activity. In contrast, even low AOM doses caused significantly enhanced O6-MeG levels in Mgmt−/− animals. The O6-MeG levels mirrored the dose-dependent CRC formation in WT and DNA repair defective animals.

**NOC activate DDR in colorectal tissue with increased γ-H2AX levels in Mgmt−/− mice**

We characterized the NOC-induced DDR in colorectal tissue of mice that differ in their DNA repair status. The DS8 marker γ-H2AX was determined in situ 24 h after AOM administration. AOM triggered dose-dependent formation of γ-H2AX foci in WT animals as visualized by confocal microscopy (Figure 4A). Induction of γ-H2AX was found primarily at the basal part of the colon crypts and colocalized with fast-proliferating compartments as attested by PCNA staining (Figure 4B). Importantly, AOM-induced γ-H2AX foci were also detected in colon stem cells (Figure 4C), which express the stem cell marker Lgr5 (28). Quantitative comparison of γ-H2AX levels in all genotypes by western blot revealed markedly higher levels of γ-H2AX in Mgmt−/− and DKO mice as compared to WT animals, while Aag−/− animals displayed a moderate increase (Figure 4D and Supplementary Figure 4C, available at Carcinogenesis Online). The kinetics of γ-H2AX formation in response to AOM was then monitored in liver and colorectal tissue. All genotypes displayed an increase in γ-H2AX in colorectal tissue after 24 h at 5 mg/kg BW. Thereafter, γ-H2AX levels appeared to decrease in WT, but not in DNA repair defective mice (Figure 4E and, for quantification, Figure 4F). In vivo, γ-H2AX levels returned almost to baseline in WT mice after 48 h, whereas knockout animals showed partial or no reduction in γ-H2AX, indicating the persistence of DNA damage (Supplementary Figure 4D, available at Carcinogenesis Online).

Next, the activation of the phosphoinositide 3-kinase (PI3K)-related kinases ATM and ATR, which orchestrate the DDR, was monitored (after validation of antibodies in mouse 3T3 cells; Supplementary Figure 5A, available at Carcinogenesis Online). We observed a time-dependent phosphorylation of ATR in the colorectal tissue of Mgmt−/− and DKO mice after AOM administration, whereas an increase was almost undetectable in WT and Aag−/− mice (Figure 5A and Supplementary Figure 5B, available at Carcinogenesis Online). In line with the observed ATR activation, AOM induced the phosphorylation of the ATR substrate Chk1, which was more pronounced in MGMT-deficient animals (Figure 5B and Supplementary Figure 5C, available at Carcinogenesis Online). We then determined the p53 levels and found an AOM-dependent accumulation of p53 in all genotypes. Interestingly, in the WT animals p53 declined after 48 h, while in Mgmt−/− mice it remained at high level (Figure 5C and Supplementary Figure 5D, available at Carcinogenesis Online). Phosphorylation of ATM and Chk2 was not clearly detectable in colon extracts of AOM-treated animals (data not shown). In conclusion, we have shown that AOM triggers the ATR-Chk1-p53 axis in colorectal tissue and dose-dependently induce γ-H2AX foci primarily in basal colon crypts and Lgr5-positive colon stem cells, which accumulated in Mgmt-deficient mice. The data support the notion that γ-H2AX is an early and robust marker of genotoxic stress contributing to carcinogenesis in the large intestine.

**MGMT-deficient mice are prone to NOC-induced apoptosis in basal colonocytes**

Since apoptosis was shown to counteract carcinogenesis, we monitored the level of apoptosis following AOM by TUNEL staining and confocal microscopy in colorectal tissue. WT and Aag−/− animals showed low numbers of apoptotic cells after treatment with 5 mg/kg BW for 48 h (Figure 5D and E). In Mgmt−/− and DKO mice, however, a pronounced increase in TUNEL-positive cells was detected, which was almost exclusively located at the basal colon crypts (Figure 5D and E). In MGMT-deficient mice, apoptotic cell death was clearly detectable at 3 mg/kg BW, but did not increase further at 10 mg/kg BW (Supplementary Figure 5E and F, available at Carcinogenesis Online). To validate that γ-H2AX is a marker for AOM-induced DNA strand breaks, and not for apoptotic cells, we performed γ-H2AX and TUNEL double staining (Figure 5F). Confocal microscopy showed that cells with discrete γ-H2AX foci (purple) and TUNEL-positive cells (green) did not overlap, corroborating that γ-H2AX is an early marker for NOC-induced genotoxic stress in mouse colorectal tissue and not merely a result of DNA fragmentation resulting from caspase-activated DNase during apoptosis. Taken together, mice lacking MGMT (Mgmt−/− and DKO, respectively) are highly susceptible to NOC-induced apoptosis in colon crypt cells.

### Discussion

Whether or not DNA repair causes a non-linear dose–response and, therefore, a threshold in chemically induced carcinogenesis is a long-standing open question. This issue has significant impact on our understanding of DNA repair as a barrier against endogenously formed, environmental and dietary genotoxins. In this study, we examined NOC-induced colorectal carcinogenesis in mice that differ in their DNA repair status, with particular emphasis on dose–responses. We demonstrate for the first time a non-linear dose–response in CRC following treatment with the colonotropic carcinogen AOM in DNA repair-proficient WT animals, which was corroborated by hockey stick modeling. The calculated ID of 3.5 mg/kg BW is consistent with the depletion of MGMT activity and concomitant persistence of O6-MeG DNA adducts. Interestingly, the inactivation of MGMT in colon was delayed compared to liver, which is probably attributable to the AOM metabolism, i.e. metabolic activation in the liver followed by activation in the gut (19). MGMT was completely inhibited at a dose of 10 mg/kg BW. Nevertheless, 5 mg/kg BW was sufficient to significantly increase CRC formation in WT animals despite
residual MGMT activity. Interestingly, MGMT activity was shown to vary along the gastrointestinal tract (29) and local depletion of MGMT may be sufficient for an increase in the level of O₆-MeG following NOC exposure. The local MGMT inactivation is also highlighted by the concept of ‘field cancerization’, in which a defined region of the gastrointestinal tract contains initiating tumor cells with inactivated MGMT and O₆-MeG-induced onco-

genic mutations, thereby driving CRC formation in humans (30).

Aag⁻/⁻ mice also displayed a non-linear dose–response in alkylating-induced CRC with a td of 2.9 mg/kg BW, which is slightly below that of WT animals. Intriguingly, at higher doses of 5 and 7.5 mg/kg BW, Aag⁻/⁻ animals exhibited twice as many tumors as WT animals, although they displayed a comparable amount of O₆-MeG adducts. This is consistent with the notion that lesions repaired by AAG contribute to NOC-induced colorectal carcinogenesis. Lack of AAG results in the accumulation of N7-MeG and N3-MeA adducts, which constitute the bulk of DNA lesions generated by alkylating agents. These adducts bear some mutagenic potential, which is however significantly lower than that of O₆-MeG (31). The major adduct N7-MeG was shown to delay polymerase β (Pol β)-mediated catalysis without affecting the accuracy of nucleotide incorporation (32). In contrast, N3-MeA is a minor groove DNA lesion that strongly blocks replicative polymerases. It can be bypassed by translesion polymerases ι (Pol ι) and κ (Pol κ) in a predominantly error-free manner (33). However, it is known that N3-MeA adducts are toxic and capable of inducing sister chromatid exchanges, chromosomal aberrations and cell death (34). The data suggest that the elevated tumor numbers observed in Aag⁻/⁻ mice after administration of higher AOM doses (≥ 5 mg/kg BW) are probably a result of replication blocking N-methylated purine adducts. These can cause DSBs and genomic instability, resulting from collapse of

Figure 4. AOM-triggered DDR in DNA repair-proficient and -deficient mice. (A) Dose-dependent γ-H2AX formation in colorectal tissue. WT mice were challenged with increasing doses of AOM (0, 5 and 10 mg/kg BW) for 24 h. γ-H2AX is depicted in green, whereas nuclei are shown in blue. Representative images are shown. (B) Costaining of γ-H2AX (green) and the proliferation marker PCNA (red) in colorectal tissue of WT animals treated with 10 mg AOM/kg bw. Representative images are shown. (C) Colocalization of Lgr5-positive colon stem cells and AOM-induced γ-H2AX foci in WT mice. Representative images are depicted. Green staining, Lgr5 located at the cell membrane; nuclear γ-H2AX foci are depicted in red. (D) Quantitative assessment of γ-H2AX formation in colorectal tissue of DNA repair-proficient and -deficient mice. Animals were treated with 10 mg AOM/kg BW (n = 3 per genotype) for 24 h. γ-H2AX levels were determined by western blot analysis of colorectal tissue extracts and quantified in relation to the loading control Hsp90. Data are shown as mean ± SEM. Statistical analysis was performed using unpaired, two-tailed Student’s t-test. **P < 0.01, n.s. not significant. (E) Time course of γ-H2AX formation in colon and liver of DNA repair-proficient and -deficient mice after treatment with 5 mg AOM/kg BW. γ-H2AX was detected by western blot analysis. Hsp90 served as loading control. (F) Quantitative evaluation of three independent experiments (n = 3 per time point and genotype). Colonic γ-H2AX levels were determined in relation to the loading control Hsp90 and presented as mean ± SEM.
stalled replication forks. According to this model, we hypothesize that genomic changes (e.g. chromosomal aberrations) induced by N-alkylations require a td, while point mutations triggered by $O_6$-MeG accrue in a linear way.

Our studies with *Mgmt*−/− mice demonstrated a linear dose–response in tumor formation without a threshold. The data highlight the pivotal role of MGMT as a barrier against NOC-induced CRC at low dose levels. The linear increase in AOM-induced tumors correlated well with the generation of hepatic $O_6$-MeG adducts. Interestingly, *Mgmt*−/− mice exhibited a similar tumor number as *Aag*−/− animals at 5 mg AOM/kg BW. This may be explained by the fact that basal colonocytes of *Mgmt*−/− animals, in which $O_6$-MeG is persistent in DNA, are prone to undergo apoptosis, as shown here and previously (8,9), to limit tumor formation at high AOM doses. Another interesting aspect revealed by the dose–response experiments is that WT mice at higher AOM doses (>3 mg/kg BW) parallel CRC formation in *Mgmt*−/− mice. We hypothesize that this is due to MGMT inactivation resulting from the repair of $O_6$-MeG adducts that causes a MGMT-deficient phenotype at high dose levels, which is illustrated by the shift of the dose–response curve with comparable slopes. Mice deficient for both *Mgmt* and *Aag* (DKO) displayed a linear dose–response in NOC-induced CRC formation similar to *Mgmt*−/− animals, confirming that MGMT is the critical protecting node in NOC-induced colorectal carcinogenesis. Of note, DKO mice did not show additive or synergistic tumor formation at 5 mg AOM/kg BW if compared with *Aag*−/− and *Mgmt*−/− animals. This might be attributable to enhanced cytotoxicity of AOM-induced lesions in critical targets in DKO mice, which attenuates tumor formation. We were not able to show increased apoptosis levels in DKO mice, but observed higher lethality rates in DKO versus *Mgmt*−/− mice.

Previous studies showed non-linear dose–responses for genotoxicity and mutagenicity in response to alkylating agents, which was attributed to DNA repair as an underlying mechanism that protects against mutation induction at low doses (15,17,18,35). However, no data is available on DNA repair-deficient experimental models with cancer formation as endpoint. Our study demonstrates a non-linear dose–response in NOC-induced colorectal tumor formation. Lack of AAG did not affect non-linearity, but tremendously enhanced tumor formation at higher doses as compared to WT mice. Obviously, at low dose levels non-repaired N-methyl adducts can be tolerated and do not give rise to tumor formation. In contrast, mice deficient for MGMT displayed a linear dose–response of alkylation-induced CRC formation and loss of a carcinogenic td. This indicates that, in contrast to N-methylations, low amounts of unrepaired $O_6$-MeG adducts are not tolerated, giving rise to tumor formation. The data support the concept that MGMT is a key node in the defense against mutations and cancer. In line with this it has been shown that MGMT
overexpressing transgenic mice have significantly enhanced tolerance to N-methyl-N-nitrosourea-induced skin papillomas with an enhanced point of departure (36). Having demonstrated a key role for MGMT in the defense against NOC-induced colorectal carcinogenesis, the NOC-induced DDR in colorectal tissue was studied to identify early markers of genotoxic stress preceding carcinogenesis. We found that AOM induced γ-H2AX in a dose-dependent manner preferentially in basal colon crypts, including stem cells characterized by Lgr5 expression (28). Interestingly, MGMT-deficient mice (Mgmt−/− and DKO) showed the highest levels of γ-H2AX, which was primary attributable to the increased levels of O-6-MeG adducts compared to WT mice. Of note, Aag−/− mice displayed moderately higher γ-H2AX levels as WT mice, indicating the contribution of non-repaired N-methylated purines. Persistent DNA damage in crypt stem cells most probably results in the fixation of mutations, thereby turning these cells into tumor-initiating cells, which are thought to trigger the ‘bottom-up’ pathway of CRC formation (37,38). Alternatively, colorectal carcinogenesis can be initiated in a ‘top-down’ model by non-stem cells with acquired stem cell capacity due to strongly elevated Wnt signaling (38). The induction of γ-H2AX was associated with AOM-dependent activation of the ATR-Chk1-p53 axis in all mouse strains tested, albeit to different extent and kinetics. Both persistent O-6-MeG adducts and N-methylated purines are likely to have contributed to the DDR activation. On the one hand, O-6-MeG mispaired with thymine engages the MMR system, which leads to strand breaks and subsequent replication fork collapse (39), and also directly activates the ATR/ATRIP signaling cascade (40). On the other hand, N-methylated purines, in particular N3-MeA (33), are capable of blocking replication and thereby trigger ATR signaling. We further demonstrate that Mgmt−/− and DKO mice challenged with AOM doses ≥3 mg/kg BW displayed strongly enhanced apoptosis in colon crypt cells. Importantly, apoptotic cells revealed by TUNEL staining did not overlap with γ-H2AX-positive cells, emphasizing the suitability of γ-H2AX as an early marker of genotoxic stress in the large intestine. Whether γ-H2AX is an early marker of colon carcinogenesis induced by NOCs remains to be seen.

Taken together, this study demonstrates for the first time a non-linear dose–response in NOC-induced carcinogenesis and reveals MGMT as the critical player that causes a carcinogenic threshold in response to 0.1 alkylating agents. AAG-mediated BER also protects against alkylated-induced CRC, which was obvious at higher AOM doses. The lack of a threshold in AAG-deficient mice might be explained by the lower mutagenicity of N-alkylations and their tolerance at low dose levels as compared to O-6-MeG adducts. The reported findings have tremendous impact on human health protection and risk assessment of chemicals and pharmaceuticals, in which safe dose and threshold levels are calculated based on ‘no observed adverse effect levels’ and uncertainty factors accounting for inter-species and inter-individual differences. It is known that MGMT activity levels in peripheral blood lymphocytes differ up to 8-fold between individuals (41). Consistent with this finding, the inter-individual variation of MGMT activity in normal colorectal tissue was up to 6-fold (42) and is also affected by longitudinal changes in the large intestine (29). This highlights the need of taking into account the inter-individual variability of MGMT, if safe dose levels should be derived for S1-alkylating agents such as N-nitrosamines found in the diet. Finally, dose–response studies with other DNA repair knockout models on dietary and environmental carcinogens are warranted in order to determine how and to what extent DNA repair confers protection against cancer formation at low dose levels.

Supplementary material

Supplementary Table 1 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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