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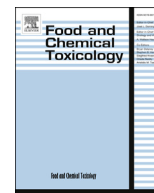

Impact of DNA repair on the dose-response of colorectal cancer formation induced by dietary carcinogens

Jörg Fahrer*, Bernd Kaina*

Department of Toxicology, University Medical Center Mainz, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany

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ABSTRACT

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers, which is causally linked to dietary habits, notably the intake of processed and red meat. Processed and red meat contain dietary carcinogens, including heterocyclic aromatic amines (HCAs) and *N*-nitroso compounds (NOC). NOC are agents that induce various N-methylated DNA adducts and O^6-methylguanine (O^6-MeG), which are removed by base excision repair (BER) and O^6-methylguanine-DNA methyltransferase (MGMT), respectively. HCAs such as the highly mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) cause bulky DNA adducts, which are removed from DNA by nucleotide excision repair (NER). Both O^6-MeG and HCA-induced DNA adducts are linked to the occurrence of *KRAS* and *APC* mutations in colorectal tumors of rodents and humans, thereby driving CRC initiation and progression. In this review, we focus on DNA repair pathways removing DNA lesions induced by NOC and HCA and assess their role in protecting against mutagenicity and carcinogenicity in the large intestine. We further discuss the impact of DNA repair on the dose-response relationship in colorectal carcinogenesis in view of recent studies, demonstrating the existence of 'no effect' point of departures (PoDs), *i.e.* thresholds for genotoxicity and carcinogenicity. The available data support the threshold concept for NOC with DNA repair being causally involved.

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1. Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers worldwide with about 1.4 million new cases in 2012 (Ferlay et al., 2015). Numerous risk factors are known to predispose to CRC formation, including genetic defects, non-dietary factors and dietary habits. It has been estimated that over 30% of all CRC cases are attributable to the diet (Parkin et al., 2011). In particular, a low intake of dietary fiber and a high consumption of red and processed meat are causally linked to increased CRC formation (Bingham et al., 2003; Norat et al., 2005). Very recently, the International Agency for Research on Cancer (IARC) in Lyon classified processed meat as carcinogenic to humans (group 1) and red meat as probably carcinogenic to humans (group 2A) (Bouvard et al., 2015). Red meat is muscle meat with a high content of myoglobin, such as beef, pork and lamb, which was not physically or chemically processed. In turn, processed meat is defined as meat treated by salting, curing, heating or smoking in order to improve its microbiological stability or flavor. Processed meat such as bacon, sausage and hot dogs may contain red meat (e.g. beef) and/or white meat (e.g. poultry) as well as meat byproducts such as blood. The processing of meat and its subsequent cooking at high temperatures, such as baking, frying and grilling, give rise to food-borne carcinogens (Sugimura, 2000). These compounds include polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines (HCAs) and *N*-nitroso compounds (NOC), which cause DNA damage following metabolic activation. The two latter groups of dietary carcinogens are of particular interest in colorectal carcinogenesis, whereas PAHs also target various other tissues. Red meat contains high levels of heme-iron, which is the prosthetic group of myoglobin involved in oxygen transport. Heme-iron bears genotoxic potential through promoting the formation of alkylating NOC and reactive oxygen species in the colorectum (Santarelli et al., 2008).

Several DNA repair pathways are involved in the protection against these DNA-damaging dietary carcinogens. Recent studies provided evidence that DNA repair is a fundamental barrier conferring resistance towards colorectal cancer formation,

* Corresponding authors. Department of Toxicology, University Medical Center Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany.
E-mail addresses: fahrer@uni-mainz.de (J. Fahrer), kaina@uni-mainz.de (B. Kaina).

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accounting for the existence of operative dose thresholds (Guerard et al., 2015), which will be detailed in the following sections.

2. Heterocyclic aromatic amines

2.1. Formation, metabolic activation and DNA adduction

HCAs arise during the cooking of meat at high temperature. They can be separated into two classes, the amino-imidazoarenes (AIA) and pyrolytic HCAs. The latter group is generated by pyrolysis of certain amino acids (*e.g.* glutamic acid and tryptophan) at temperatures above 250 °C, which gives rise, among others, to the glutamic acid pyrolysate 2-amino-6-methyldypyrido[1,2-*a*:30,20-*d*] imidazole (Glu-P-1) and the tryptophan pyrolysate 2-amino-1,4-dimethyl-5Hpyrido[4,3-*b*]indole (Trp-P-1) (Skog et al., 1997, 1998). The group of AIAs is formed in meat cooked at lower temperatures (150–250 °C) due to the Maillard reaction, involving creatin(in)e and Strecker degradation products (Jagerstad et al., 1991). Important representatives of this class include 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ), 2-amino-3-methylimidazo [4,5-f]quinoxaline (IQx) and 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP). PhIP is the most abundantly formed HCA with levels up to 500 ppb found in cooked meat (Ni et al., 2008; Sinha et al., 1995). It was further demonstrated that PhIP can be produced *in vitro* by heating a mixture of creatine, phenylalanine and glucose (Skog and Jagerstad, 1991).

HCAs require metabolic transformation to DNA-reactive ultimate carcinogens, which involves N-hydroxylation at their exocyclic amino group followed by esterification leading to a sulfate or acetate group (Fig. 1). PhIP is firstly converted to *N*-hydroxy-PhIP (N-OH-PhIP) by cytochrome P450 (CYP) 1A2 during phase I metabolism and then mainly conjugated with via 3'-phosphoadenosine-5'-phosphosulfate (PAPS) by sulfotransferase (SULT) 1A1 in phase II (Fig. 1A), since it is a poor substrate for N-acetyltransferases (NATs) (Muckel et al., 2002; Sugamori et al., 2006). In contrast, IQ and MeIQx undergo efficient O-acetylation catalyzed by NAT2, leading to the formation of *N*-acetoxy intermediates that covalently bind to DNA (Metry et al., 2009; Wu et al., 1997). The primary site of HCA adduct formation is the C8-atom of guanine in DNA, while it occurs less frequently at the *N*2-position of guanine. The major DNA adduct of PhIP is dG-C8-PhiP (Lin et al., 1992), which is found at high levels in extrahepatic tissues, including colorectal and mammary tissue (Ghoshal et al., 1995; Kaderlik et al., 1994a). The low levels in the liver are attributed to an efficient detoxification of N-OH-PhIP via conjugation with glucuronic acid and glutathione (Kaderlik et al., 1994b; Lin et al., 1994). The dG-C8-PhIP adduct levels measured in human tissues (*e.g.* breast, prostate, etc.) are in the range of those detected in the corresponding tissue of rodents challenged with PhIP doses up to 50 mg/kg bw (Kaderlik et al., 1994a; Shirai et al., 1997; Tang et al., 2007; Zhu et al., 2003), implying that PhIP DNA adduct formation occurs in highly efficient manner in humans. IQ generates preferentially dG-C8-IQ adducts, but also reacts with the *N*2-atom of guanine yielding dG-N2-IQ adducts (Jamin et al., 2007; Turesky et al., 1992). This is attributable to the existence of two activated IQ species, an arylnitrenium ion and an arylcarbenium ion (Fig. 1B). IQ-DNA adducts were found both in liver and in colon tissue of rats fed with a diet containing IQ as revealed by ^{32}P-postlabeling technique (Moller et al., 2002). Interestingly, the structurally related MeIQ was shown to induce tenfold more dG-C8-MeIQ adducts in the liver than in the colon of mice that received a supplemented diet for 4 weeks (Kim et al., 2016). Taken together, HCAs require metabolic activation by phase I and II enzymes to form the ultimate DNA reactive species that damage DNA primarily at the C8-position of dG and, to a lesser extent, at the *N*2-position of dG.

2.2. Removal of HCA-DNA adducts by nucleotide excision repair

As pointed out above, HCAs such as PhIP induce bulky DNA lesions primarily at dG moieties, which results in a distortion of the DNA double helix (Brown et al., 2001). This type of DNA damage is recognized by proteins initiating the nucleotide excision repair (NER) pathway, which is a sophisticated process involving more than 25 different repair factors (Iyama and Wilson, 2013). NER is also involved in the removal of adducts generated by the anticancer drug cisplatin, the chemical carcinogen benzo[*a*]pyrene and UV radiation (Gillet and Schärer, 2006). Due to their size and helix-distorting property, these DNA lesions can impede both DNA replication and DNA transcription. There are two existing routes of NER, which follow common repair steps, but differ in their mode of damage recognition. In global genome repair (GG-NER), the damage is removed throughout the genome, whereas transcription-coupled repair (TC-NER) is engaged by lesions on the transcribed strand of DNA, interfering with RNA polymerase progression (Fousteri and Mullenders, 2008; Gillet and Schärer, 2006).

As illustrated in Fig. 2A, GG-NER is initiated by a dimer consisting of xeroderma pigmentosum protein C (XPC) and RAD23B, which recognizes the DNA damage-induced alteration of the helical DNA structure (Maillard et al., 2007; Min and Pavletich, 2007). XPC-RAD23B then recruits transcription factor II H (TFIIH) complex, which comprises, among other factors, the two helicases XPB and XPD (Sugasawa et al., 2009; Yokoi et al., 2000). TFIIH catalyzes the unwinding of DNA around the lesion in 3′–5′ (XPB) and 5′–3′ (XPD) direction in an ATP-dependent manner (Oksenych and Coin, 2010), thereby generating a “bubble”. This structure serves as binding platform for XPA and replication protein A (RPA), forming the pre-incision complex. XPA is responsible for the release of TFIIH and promotes the coating of single-stranded damaged DNA with RPA (Coin et al., 2008; Li et al., 1995; Missura et al., 2001). In a next step, the XPF-excision repair cross complementing 1 (ERCC1) complex is located to the lesion via binding to XPA, while XPG is recruited by TFIIH. These two endonucleases catalyze the incision 5′ and 3′ to the DNA damage (Staresincic et al., 2009), leading to the release of an oligonucleotide spanning 10–15 bases with the damaged base. The gap is then filled by the activity of DNA polymerase δ or ε, which cooperate with replication factor C (RFC) and proliferating cellular nuclear antigen (PCNA) (Ogi et al., 2010; Overmeer et al., 2010). Finally, a complex of X-ray repair cross-complementing protein 1 (XRCC1) and DNA ligase III (LIG3) or flap endonuclease 1 (FEN1) and DNA ligase I (LIG1) restores the DNA phosphodiester backbone by sealing the nick (Moser et al., 2007). In contrast to GG-NER, the TC-NER pathway is triggered by stalled RNA polymerase (RNAP) on the transcribed strand within an active gene. This process involves Cockayne syndrome (CS) proteins, CSA and CSB, which are recruited to the blocked RNAP, thereby facilitating the recruitment of the canonical NER factors and subsequent removal of the damaged site (Fousteri and Mullenders, 2008).

Major evidence for NER-dependent removal of HCA-DNA adducts stems from *in vivo* experiments, whereas *in vitro* and cell culture studies dealing with the repair of HCA-induced DNA adducts are quite rare. It has been shown *in vitro* that dG-C8-PhIP adducts present in a deletion duplex (*i.e.* the dC on the complementary DNA strand is missing) are repaired with an efficiency of only 15–20% as compared to the full duplex, which was attributed to an impaired XPC binding and recruitment of downstream NER factors (Reeves et al., 2011). A deletion duplex may be formed during DNA replication, if the replicative DNA polymerase fails to incorporate a dNTP opposite to the bulky adduct, but instead continues with the next template base downstream. A previous study in rats addressed the adduct kinetics over a period of 5 weeks upon initial PhIP administration, and revealed that still 25% of the

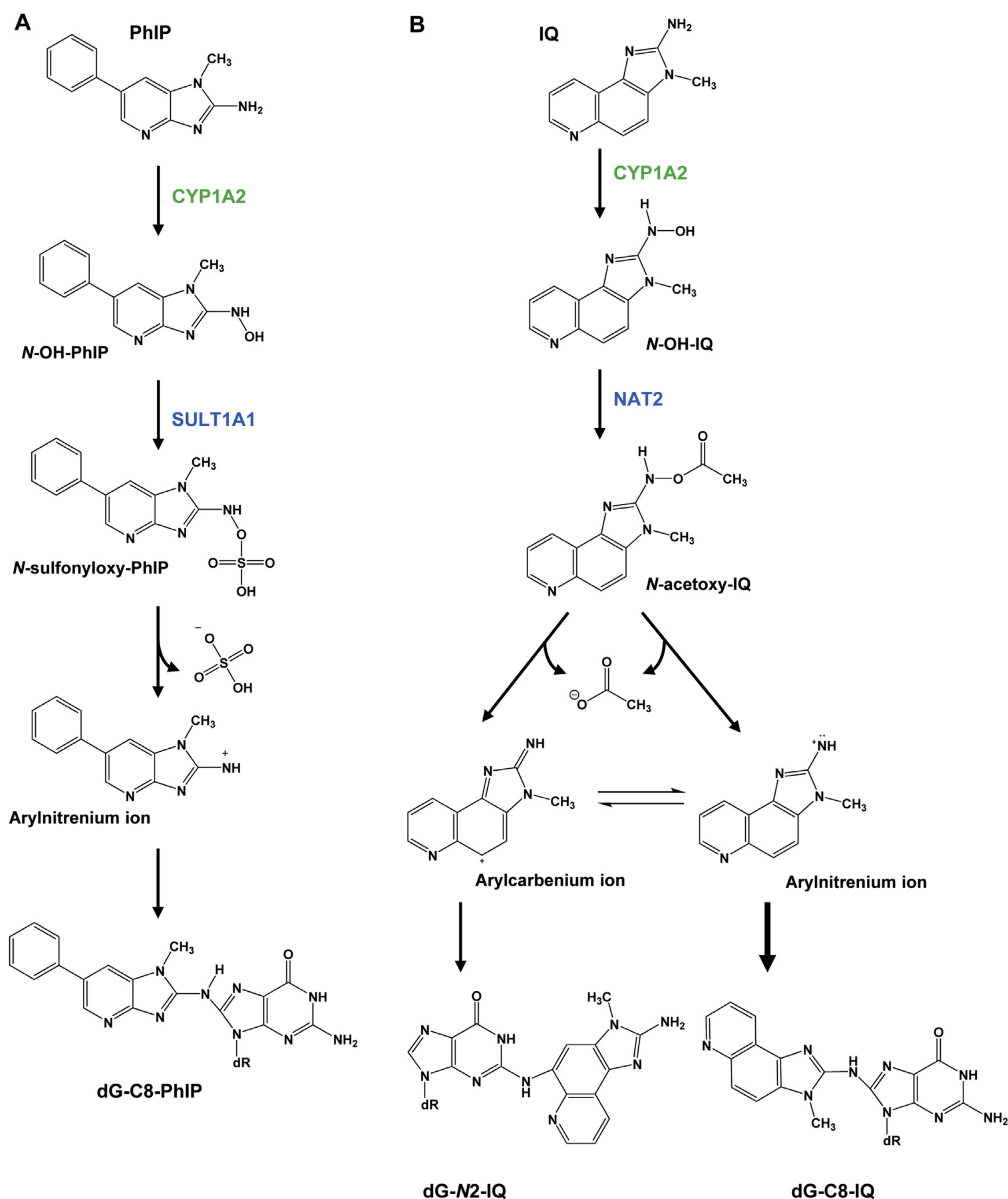

Fig. 1. Metabolic activation of HCA and DNA adduct formation. A PhIP is activated by CYP1A2, yielding the toxic metabolite *N*-OH-PhIP. *N*-OH-PhIP is then conjugated by SULT1A1, giving rise to *N*-sulfonyloxy-PhIP. Due to its instability, a DNA-reactive arylnitrenium ion is released, which generates primarily C8-PhIP-dG adducts. **B** Similar to PhIP, IQ is converted to *N*-OH-IQ by CYP1A2 followed by its acetylation mediated by NAT2. The formed *N*-acetoxy-IQ is instable and gives rise to both an arylnitrenium ion and an arylcarbonium ion. These react with DNA to form mostly dG-C8-IQ and dG-*N*2-IQ DNA adducts. CYP: cytochrome P450; SULT: sulfotransferase; NAT: N-acetyltransferase.

initially induced DNA adducts were present after one week, suggesting a slow repair rate (Ghoshal et al., 1995). Further i*n vivo* experiments using XPA-defective mice provided evidence that PhIP-DNA adducts are substrates of the NER pathway. Following short term oral PhIP administration, $XPA^{-/-}$ animals displayed twice as high DNA adduct levels in liver, colon and lung as compared to wild-ype (wt) animals, which was determined by ^{32}P-postlabeling (Imaida et al., 2000). The removal of PhIP-DNA adducts was retarded in $XPA^{-/-}$ animals, most likely due to compromised NER (Imaida et al., 2000). PhIP was shown to be highly toxic in $XPA^{-/-}$ mice, which was reflected in rapid weight loss and intestinal abnormalities (Klein et al., 2001). In contrast, no toxicity was observed in animals lacking XPC, which retain the capability for transcription-coupled NER, but are defective in the initiation of global genomic NER (Klein et al., 2001). This finding indicates that the removal of PhIP-DNA adducts from actively transcribed genes by TC-NER is essential to prevent intestinal toxicity. Moreover, an increased mutation frequency was observed in the *lacZ* reporter gene in intestinal and liver DNA of $XPA^{-/-}$ animals exposed to low doses of PhIP (Klein et al., 2001), providing evidence that

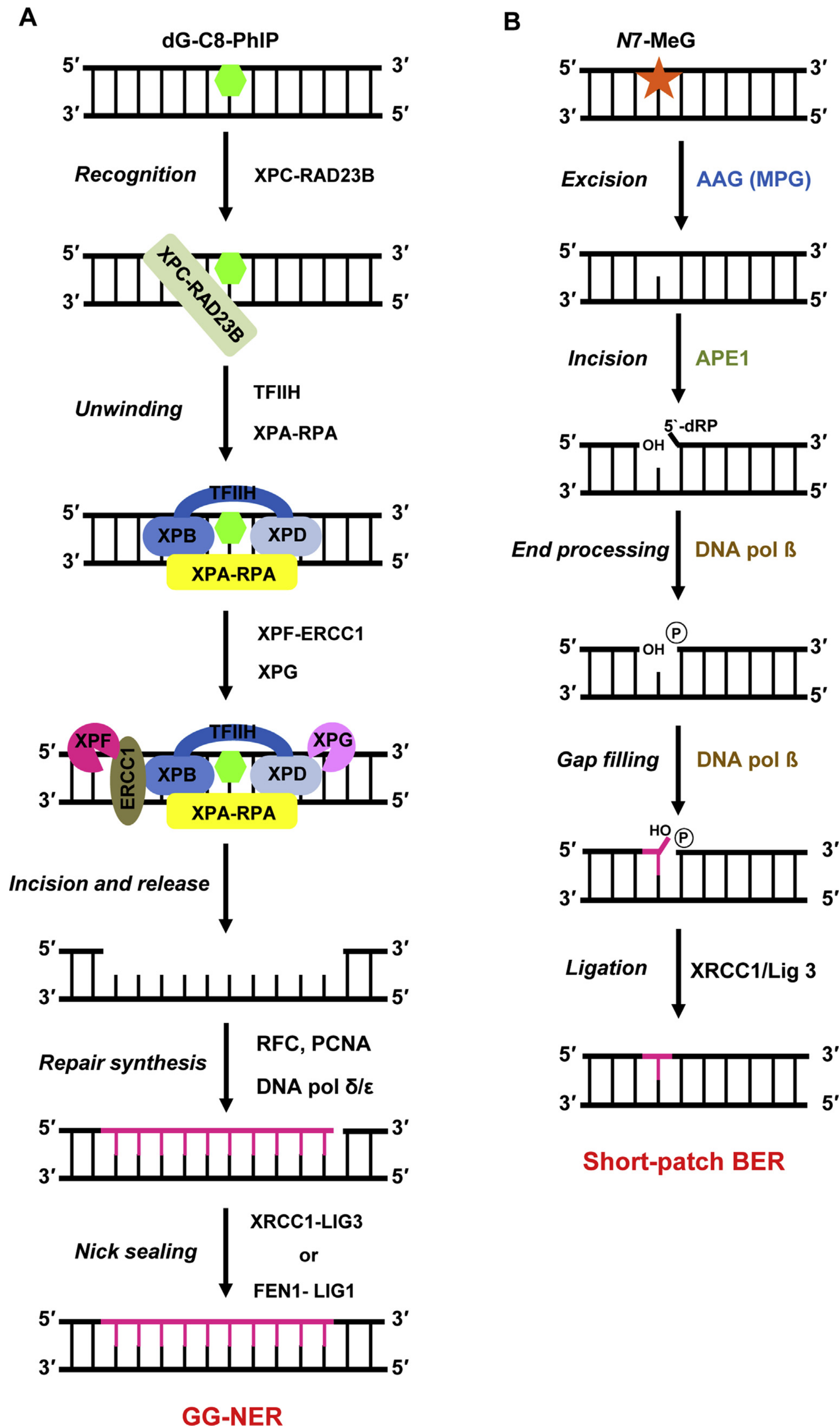

Fig. 2. Mechanism of nucleotide excision repair (NER) and base excision repair (BER). A Bulky adducts including C8-PhIP-dG undergo NER. In global genomic (GG)-NER, the helix-distorting lesion is recognized by the XPC-RAD23B dimer, whereas transcription-coupled (TC)-NER is triggered by stalled RNA polymerase on the transcribed strand within an active gene. This process involves Cockayne syndrome (CS) proteins, CSA and CSB. Following this initial step, both pathways converge and proceed involving the same key repair factors. **B** BER is a highly conserved pathway that removes damaged DNA bases. The N-alkylation adducts *N*7-MeG and *N*3-MeA are recognized by the DNA glycosylase AAG

unrepaired PhIP-DNA adducts are mutagenic. Another study analyzed the role of the *Adenomatous polyposis coli* (*APC*) tumor suppressor gene in PhIP-induced DNA adduction and removal. Interestingly, heterozygous $APC^{+/-}$ mice on a XPA-deficient background displayed a higher DNA adduct level in different tissues including liver and small intestine than their wt counterparts (Steffensen et al., 2006). This was confirmed in another study using established murine intestinal cells obtained from $APC^{+/-}$ mice, which showed a higher DNA adduct level and increased formation of phosphorylated histone 2AX (γ-H2AX) as compared to $APC^{+/+}$ cells (Jamin et al., 2013). Collectively, the available data shows that HCA-DNA adducts, including dG-C8-PhIP, are helix-distorting DNA lesions that are subject to NER. If left unrepaired, these adducts give rise to mutations, finally leading to cancer formation as discussed below.

2.3. Mutagenicity and carcinogenicity of HCAs in the large intestine

HCAs were shown to be mutagenic in various bacterial and mammalian test systems (David et al., 2015; Muckel et al., 2002; Yadollahi-Farsani et al., 1996). This was corroborated in animal studies performed in rodents. Colon tumors isolated from rats fed with a diet containing 0.04% PhIP harbored deletion mutations within G stretches such as 5′-GGGA-3′ in the *APC gene*, leading to a truncated APC protein (Kakiuchi et al., 1995). Conversely, tumors with wild type APC exhibited mutations in codons 32 and 34 of *ß-catenin,* including G → A and G → T mutations (Dashwood et al., 1998). This was confirmed in another study, in which ICR (established at the Institute for Cancer Research in Philadelphia; also referred to as Crl:CD1) mice received a single oral dose of 200 mg PhIP/kg bw followed by the administration of the colon-specific tumor promoter dextran sodium sulfate (DSS) (Tanaka et al., 2005). Isolated colonic tumors had mutations limited to codon 32 and 34 of *ß-catenin*, involving primarily G → A mutations. Further studies in genetically engineered mice with human CYP1A1 and CYP1A2 expression revealed predominantly mutations in codon 32 and 34 of *ß-catenin* in colonic tumors by whole exome sequencing (Wang et al., 2015). Strikingly, no mutations were found in *KRAS* (Kirsten rat sarcoma viral oncogene homolog) and *APC,* indicating that *ß-catenin* mutations are critical drivers in PhIP/DSS-triggered CRC. Interestingly, CYP1A-humanized mice rapidly developed multiple colorectal tumors within 6 weeks upon exposure to PhIP followed by DSS, whereas in wild-type animals no tumor formation was observed, highlighting the important species-specific differences in CYP1A-dependent PhIP metabolism (Cheung et al., 2011). PhIP was further shown to induce a higher colorectal tumor incidence and multiplicity than the other AIAs IQ or MeIQx, using a diet enriched with 300 ppm PhIP in combination with 2 cycles of DSS (Nishikawa et al., 2005).

Both IQ and MeIQx exert their carcinogenic activity preferentially in the liver (Kushida et al., 1994; Ohgaki et al., 1984), but also induce tumors in the large intestine if promoted by DSS (Nishikawa et al., 2005; Tanaka et al., 2005). Finally, chronic exposure of NER-defective $XPA^{-/-}$ animals to low dietary levels of PhIP (25 ppm) resulted in tumor formation in different organs, including the intestine (Klein et al., 2001).

Data obtained from epidemiological studies are in agreement with the carcinogenicity of PhIP and other AIAs observed in the large bowel of laboratory animals. A large case-control study demonstrated that high NAT2 activity combined with a high dietary intake of HAA (PhIP, MeIQx and DiMeIQx) is associated with an increased risk to develop colorectal adenomas (Voutsinas et al., 2013). Another recent epidemiological study confirmed the positive association between HCA intake and colorectal cancer formation (Miller et al., 2013). Given its abundance in cooked meat and the efficient metabolic activation by human CYP1A2, PhIP is of particular interest for colorectal carcinogenesis. This is underpinned by a recent study, showing that defects in the DNA damage response machinery potentiate the genotoxicity and clastogenicity of PhIP in human epithelial colonocytes (Mimmler et al., 2016).

3. *N*-nitroso compounds

3.1. Occurrence, metabolic activation and DNA adduct formation

N-nitroso compounds (NOC) are formed in a chemical reaction between nitrite or nitrogen oxides and secondary amines or *N*-alkylamides. NOC occur wide-spread in the diet and have been detected especially in processed meat, smoked fish, cheese and in beer (Lijinsky, 1999). The NOC levels in food are primarily affected by the curing process, the temperature and the amounts of secondary amines present (Behsnilian et al., 2014). Apart from the dietary sources, NOC can also arise endogenously in the stomach and the large intestine, where non-resorbed amino acids undergo bacterial decarboxylation to amines followed by their nitrosation (Fahrer and Kaina, 2013). The intestinal production of NOC is increased in the presence of high NO levels, which exist during chronic inflammation of the gut epithelium, *e.g.* in ulcerative colitis (Kimura et al., 1998). Furthermore, the intake of heme or heme-containing red meat was shown to stimulate the generation of NOC measured as apparent total nitroso compounds (ATNC) in the feces (Joosen et al., 2009; Kuhnle et al., 2007; Lewin et al., 2006). ATNC comprise *S*-nitrosothiols, nitrosyl-iron and *N*-nitrosamines, whereof nitrosyl-iron is the major constituent (Joosen et al., 2009, 2010).

The formed NOC, *i.e. N*-nitrosamines, are pro-carcinogens that require metabolic activation by CYP2E1, which catalyzes the hydroxylation of the α-C-atom (Fig. 3) (Yang et al., 1990). The generated intermediates are instable and rearrange under the release of an aldehyde and molecular nitrogen, thereby giving rise to highly reactive alkyl carbocations. These ultimate carcinogens induce a broad spectrum of alkylation damage in DNA via a S_N1 nucleophilic substitution (Fu et al., 2012). The most abundant lesions are *N*7-methylguanine (*N*7-MeG) and *N*3-methyladenine (*N*3-MeA), which represent approximately 75% of total DNA alkylation damage (Beranek, 1990). Another important DNA lesion is O^6-methylguanine (O^6-MeG), which contributes to maximally 8% of all alkylated DNA adducts (Beranek, 1990). O^6-MeG is a very critical DNA lesion due to its high mutagenic and cytotoxic potential (Kaina et al., 2007). Administration of the colonotropic carcinogen azoxymethane (AOM) or its downstream metabolite 1,2-dimethylhydrazine (DMH) to rodents was shown to induce O^6-MeG adducts in colorectal tissue as detected by immunohistochemistry (Jackson et al., 2003; Nyskohus et al., 2013). Both AOM and DMH are NOC-related compounds, which are used as tumor initiators in chemically-induced colon cancer models (Fahrer and Kaina, 2013; Neufert et al., 2007).

An increased level of colonic O^6-MeG adducts has recently been

(designated also as MPG), which removes the damaged base followed by incision at the phosphodiester backbone catalyzed by APE1. The remaining 5′-deoxyribosephosphate (5′-dRP) terminus is then eliminated by Pol β, which inserts a novel nucleotide to fill the gap. The remaining nick is finally sealed by concerted action of XRCC1 and DNA ligase III (short-patch BER). Under certain conditions, *e.g.* if the 5′-dRP moiety is chemically altered or cells are in S-phase, the repair is completed via the long-patch pathway using additional repair factors as indicated in the text.

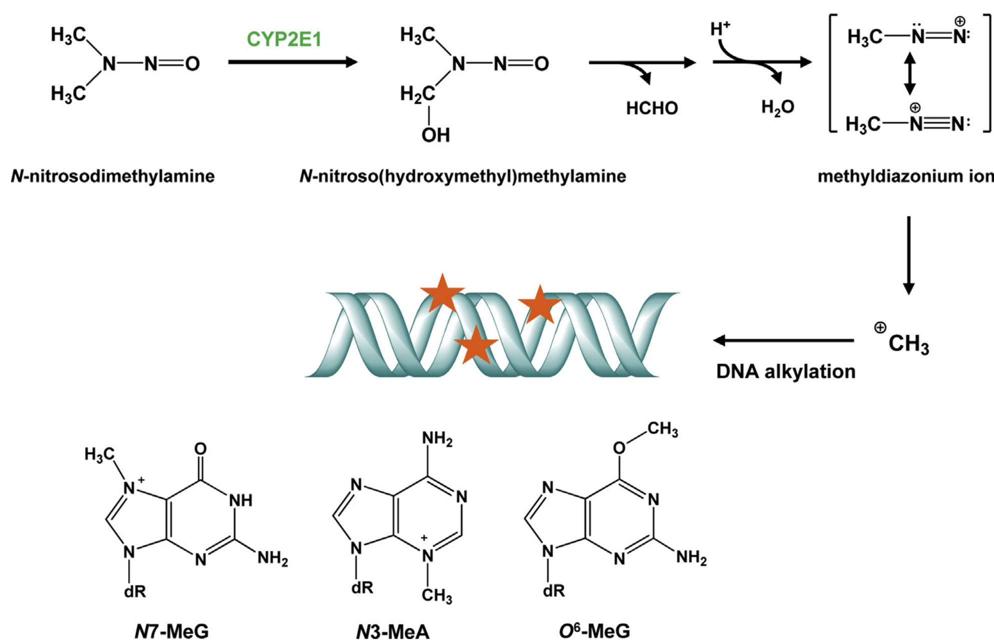

Fig. 3. Metabolic activation of NOC and DNA damage induction. NOC, here *N*-nitrosodimethylamine, are N-hydroxylated by CYP2E1. The formed *N*-nitroso(hydroxymethyl) methylamine rearranges under the release of formaldehyde and water to an instable methyldiazonium ion. This intermediate decomposes spontaneously to a methylcarbocation, which attacks the DNA, thereby inducing primarily *N*-methyl purines (*N*7-MeG and *N*3-MeA) and, as a minor product, O^6-MeG.

observed in mice, which received a red meat-containing diet (Winter et al., 2011). The control group fed with casein as protein source had lower adducts levels, suggesting that red meat may increase the formation of colonic O^6-MeG adducts. It is interesting to note that high levels of this DNA adduct were also found in normal tissue of sigmoid colon and rectum, which was obtained from patients undergoing colorectal surgery (Povey et al., 2000a). Thus, O^6-MeG appears to be a biomarker for exposures to alkylating carcinogenic species. *N*7-MeG adducts were also detected in human colon biopsies (Lees et al., 2007), but are less frequently used as a marker of alkylation DNA damage due to the lack of commercially available antibodies. Overall, the available data show that the intake of heme-containing red meat and inflammatory bowel disease promote the generation of NOC in the colorectum, which may lead to the formation of the mutagenic adduct O^6-MeG.

3.2. Repair of alkylation DNA damage by direct damage removal and base excision repair

The most important pathways involved in the repair of alkylated DNA adducts are base excision repair (BER) and direct damage reversal by O^6-methylguanine-DNA methyltransferase (MGMT). BER is a highly conserved pathway, which removes, amongst others, the main *N*-methylated DNA adducts *N*7-MeG and *N*3-MeA (Fig. 2B). The damaged bases are recognized by the *N*3-alkyladenine-DNA glycosylase (AAG; also referred to as N-methylpurine-DNA glycosylase, MPG), which catalyzes the hydrolysis of the N-glycosidic bond between the base and the deoxyribose moiety (Chakravarti et al., 1991; Engelward et al., 1997). This leads to the release of the damaged base, generating an abasic site. In a next step, AP endonuclease (APE1) catalyzes the incision of the phosphodiester backbone at the abasic site, inducing a nick with a 5′-deoxyribose-5-phosphate (5′-dRP) and a free 3′-OH group (Demple and Sung, 2005). The 5′dRP moiety is primarily eliminated by DNA polymerase β (Pol β) via its intrinsic lyase activity, producing a 5′-phosphate terminus (Beard and Wilson, 2006). BER is then typically completed via the short-patch pathway, in which Pol β catalyzes the incorporation of a new nucleotide using the 3′-OH group as primer. Finally, the remaining nick is sealed by XRCC1 and DNA ligase III (Cappelli et al., 1997). Alternatively, BER can proceed via the long-patch (LP) pathway, if the 5′-dRP moiety is chemically modified (and therefore no substrate for Pol β), during S-phase of the cell cycle and at low ATP levels (Klungland and Lindahl, 1997; Krokan and Bjoras, 2013; Petermann et al., 2003). The LP pathway involves additional factors including Pol δ and ε, PCNA, FEN1 and DNA ligase I (Svilar et al., 2011).

MGMT is a suicide DNA repair enzyme, which catalyzes the covalent transfer of the methyl group from the O^6-MeG onto a cysteine residue located in its catalytic cleft (Daniels et al., 2004; Guengerich et al., 2003) (Fig. 4). This reaction restores the guanine base in DNA, but causes inactivation of the MGMT protein and subsequent degradation via the ubiquitin/proteasome pathway (Christmann and Kaina, 2013; Srivenugopal et al., 1996). MGMT is also responsible for the removal of larger O^6-alkylguanine adducts such as O^6-chloroethylguanine induced by *N*-nitrosourea-based anticancer drugs (Preuss et al., 1996). In addition, MGMT repairs the bulky adduct O^6-[4-oxo-4-(3-pyridyl)butyl]guanine (Mijal et al., 2004), which is generated by tobacco-specific *N*-nitrosamines (Christmann and Kaina, 2012). Interestingly, the activity and protein expression of MGMT is regulated by various natural compounds. Cysteine prodrugs, natural antioxidants like curcumin and coffee diterpenes were shown to elevate MGMT levels and activity in human colorectal cancer cells *in vitro* and in rat hepatic tissue *in vivo* (Huber et al., 2003; Niture et al., 2007). Whether MGMT is upregulated in humans following genotoxic stress and dietary constituents is still an open question (Christmann and Kaina, 2013). In contrast, the disulfide compounds disulfiram, a drug used in alcohol withdrawal, and α-lipoic acid, a dietary supplement with antioxidative and potential anti-tumor activity (Dörsam and Fahrer, 2016), are potent MGMT inhibitors, causing MGMT depletion on the protein level (Göder et al., 2015; Paranjpe et al., 2014). MGMT gene expression is controlled by several transcription factors (NF-κB, SP-1, p53) and notably by its promoter CpG methylation status (Christmann and Kaina, 2013). Hypermethylation of the *MGMT*

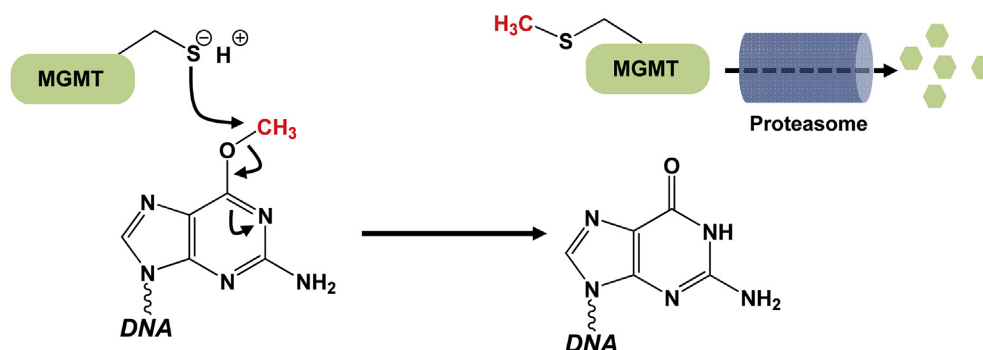

Fig. 4. Direct damage reversal by MGMT. O^6-MeG and other O^6-alkyl guanine adducts are repaired by MGMT in a stoichiometric reaction. The methyl group form O^6-MeG is transferred to a highly activated cysteine residue in the catalytic cleft of MGMT. This restores guanine in DNA and concomitantly inactivates MGMT, which is then degraded via the ubiquitin/proteasome pathway. MGMT is thus considered as suicide enzyme and has to be resynthesized upon its depletion.

promoter was reported to abrogate MGMT expression with concomitant loss of activity (Christmann et al., 2011), which has significant impact on therapy with O^6-alkylating anticancer drugs (Hegi et al., 2005). Taken together, BER is a highly conserved pathway that repairs NOC-induced N-methylated adducts in a fast and efficient manner by base excision, while MGMT specifically removes O^6-MeG and other O^6-alkylguanine adducts in a stoichiometric single-step suicide reaction. MGMT expression and activity is regulated at different levels, with *MGMT* promoter hypermethylation being of particular interest in CRC etiology.

3.3. Mutagenicity and carcinogenicity of NOC in the large intestine

O^6-MeG lesions are highly mutagenic and carcinogenic (Fu et al., 2012; Kaina et al., 2007; Pegg, 2011). If not repaired by MGMT, persisting O^6-MeG adducts mispair with thymine, resulting in G:C to A:T transition mutations following replication. The O^6-MeG:T mismatch engages the mismatch repair system (MMR), which gives rise to the formation of DNA double-strand breaks due to aberrant repair cycles, which promotes chromosomal instability, thereby contributing to cancer formation (Duckett et al., 1996; Mojas et al., 2007; Ochs and Kaina, 2000).

A first study dealing with the role of MGMT in NOC-induced mutagenicity in the colon was performed in a transgenic mouse model with overexpression of MGMT, in which a significantly reduced number of G:C to A:T transition mutations was detected in the *KRAS* oncogene upon treatment with the colonotropic alkylating agent AOM (Zaidi et al., 1995). In turn, rats treated with the pharmacological MGMT inhibitor O^6-benzylguanine (O^6-BG) displayed an increased frequency of colonic tumors harboring *KRAS* mutations following AOM exposure (15 mg/kg bw) (Wali et al., 1999). Further studies using the AOM/DSS-model and transgenic MGMT-deficient mice provided evidence that MGMT plays a pivotal role in the defense against AOM-induced colorectal cancer (Bugni et al., 2009; Wirtz et al., 2010). Animals with MGMT deficiency showed an elevated level of cells undergoing apoptosis in the colon crypts after AOM administration. This was attributable to the activation of the MMR system by persistent O^6-MeG adducts as demonstrated by lack of apoptosis induction in animals on a MGMT/MMR-defective background (Bugni et al., 2009). As expected, these double knockout animals showed higher tumor rates due to the impaired elimination of O^6-MeG bearing colon crypt cells (Bugni et al., 2009). Very recently, our studies in DNA repair competent and defective animals unveiled a very crucial role for MGMT in cancer protection at low alkylation dose levels, whereas the DNA glycosylase AAG was only relevant at high alkylation dose levels (Fahrer et al., 2015). Nevertheless, AAG also conferred resistance to inflammation-driven AOM-induced CRC, highlighting the role of N-alkylated DNA adducts such as *N*7-MeG and *N*3-MeA in colon carcinogenesis. In addition, AAG was shown to suppress colonic tumorigenesis in a setting of severe large bowel inflammation triggered by multiple cycles of 2.5% DSS in the absence of AOM initiation (Meira et al., 2008).

Apart from these animal studies, there is compelling evidence that MGMT is involved in the etiology of human colorectal cancer. About 40% of sporadic colorectal tumors display an epigenetic inactivation of *MGMT* by promoter hypermethylation, resulting in decreased MGMT expression levels (Herfarth et al., 1999; Lind et al., 2004). It was further reported that *MGMT* promoter methylation already occurred in pre-neoplastic ACF and increased with tumor progression (Chan et al., 2002; Nagasaka et al., 2008). Strikingly, loss of MGMT expression and activity has been clearly linked to an elevated *KRAS* mutation frequency in colorectal cancer (de Vogel et al., 2009; Esteller et al., 2000; Nagasaka et al., 2008). Dietary intake of heme-iron correlated with an increased risk for CRC and oncogenic G:C to A:T transition mutations in *KRAS*, again indicating the importance of O^6-alkylating agents in CRC etiology (Gilsing et al., 2013). MGMT hypermethylation was not associated with mutations in the *APC* tumor suppressor gene (de Vogel et al., 2009), which occur very early in sporadic CRC formation and are found in patients with the inherited disease familial adenomatous polyposis (FAP) (Markowitz and Bertagnolli, 2009). Taken together, loss of MGMT due to promoter hypermethylation is a critical event in the early stage of CRC development, which predisposes to oncogenic *KRAS* mutations and thereby drives tumor progression. It should be noted that studies on the role of AAG in human sporadic colorectal carcinogenesis are still lacking, which might relate to the fact that high DNA N-alkylation levels are unlikely to be found in humans, except during cancer therapy with alkylating agents. Interestingly, AAG levels were elevated in inflamed tissue obtained from patients with ulcerative colitis, which might contribute to microsatellite instability (Hofseth et al., 2003).

4. Impact of DNA repair on dose-response in CRC formation

4.1. Dose-response in NOC-induced mutagenicity and colorectal carcinogenicity

In a landmark study by Doak and co-workers, a non-linear dose response in mutagenicity was observed in lymphoblastoid cells exposed to increasing doses of the S_N2-alkylating agents methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) (Doak et al., 2007). These compounds primarily induce N-alkylated DNA bases and only very low amounts of O^6-MeG (0.3% for MMS and 2%

for EMS of total DNA alkylation products) (Beranek, 1990). The S_N1-alkylating NOC *N*-methyl-*N*-nitrosurea (MNU) and *N*-ethyl-*N*-nitrosurea (ENU), which generate 6–9% O^6-MeG adducts (Beranek, 1990), displayed a linear dose-response for gene mutations in the concentration range tested. This work provided first evidence for the existence of a 'no effect' PoD (point of departure), *i.e.* a threshold in genotoxicity, for S_N2-alkylating agents. This concept was supported by *in vivo* studies using a gene mutation test and the micronucleus test as mutagenic and clastogenic endpoints (Gocke and Muller, 2009). As observed before *in vitro*, EMS showed a non-linear dose response, which allowed for the calculation of a threshold dose using the hockey stick model (Lutz and Lutz, 2009). Furthermore, the data indicated a potential threshold for the S_N1-alkylating agent ENU at very low doses. A threshold for both MNU and MMS was determined in mouse lymphoma cells, in which mutations at the thymidine kinase locus were assessed (Pottenger et al., 2009). Further *in vivo* work in rats, which received EMS or ENU, revealed a non-linear dose-response for both DNA-reactive carcinogens using the Pig-α mutation assay, and allowed derivation of threshold doses (Dobo et al., 2011).

A potential role for MGMT in affecting the dose-dependent mutagenicity of NOC was indicated by a study in rats that received the same dose of MNU either sub-chronically or as an acute high dose (van Zeeland et al., 2008). Mutation frequency was substantially reduced after multiple treatments with low doses as compared to a single treatment with high dose, likely resulting from inactivation of MGMT at high doses (van Zeeland et al., 2008). The importance of MGMT in the protection against MNU-induced mutations was subsequently demonstrated in lymphoblastoid cells, in which depletion of MGMT by O^6-BG resulted in a 10-fold lower no-observed-genotoxic-effect-level (NOGEL) (Thomas et al., 2013). Bacterial mutagenicity assays performed in alkyltransferase proficient- and deficient *Salmonella typhimurium* strains showed a contribution of the repair catalyzed by the bacterial alkyltransferases Ogt and Ada to genotoxic thresholds observed upon treatment with alkylating agents (Tang et al., 2014). Very recently, it was demonstrated that MGMT, but not the DNA glycosylase AAG, causes a threshold in NOC-induced CRC formation at low alkylation dose levels, while both DNA repair proteins protect against NOC-induced CRC at high dose levels (Fahrer et al., 2015). In more detail, DNA repair proficient and DNA repair defective (*Mgmt*$^{-/-}$, *Aag*$^{-/-}$ and *Mgmt*$^{-/-}$/*Aag*$^{-/-}$) mice were challenged with increasing doses of AOM (0–7.5 mg/kg bw) followed by 2 cycles of DSS to trigger mild tumor promoting colitis. Mini-endoscopy revealed a non-linear dose-response of colorectal tumor formation in DNA-repair proficient and AAG-deficient animals, allowing for the calculation of a carcinogenic threshold based on the hockey stick model (Fahrer et al., 2015). In contrast, MGMT-deficient mice (*Mgmt*$^{-/-}$ and *Mgmt*$^{-/-}$/*Aag*$^{-/-}$) displayed linearity in AOM-induced colorectal carcinogenesis without a threshold. Intriguingly, O^6-MeG adduct levels and depletion of MGMT activity correlated very well with the observed tumor formation in DNA repair proficient and deficient animals (Fahrer et al., 2015). These findings unequivocally showed that DNA repair by MGMT is of crucial importance in defending against colorectal cancer initiation, thus accounting for the existence of a NOEL and threshold in NOC-triggered CRC (Fig. 5A).

In summary, the induction of O^6-MeG adducts by NOC and their subsequent repair by MGMT are of pivotal importance for colorectal carcinogenesis. In view of the variability of MGMT expression in humans (Janssen et al., 2001; Povey et al., 2000b), its modulation by natural compounds (Göder et al., 2015; Huber et al., 2003; Niture et al., 2007) and the widespread dietary occurrence of NOC, the question as to whether 'no effect' PoDs exist in NOC-induced human CRC formation warrants further studies.

4.2. Dose-response of HCA-induced colorectal and liver carcinogenesis

More than two decades ago, a first study addressed the dose-response relationship for MeIQx-induced DNA adduct formation using accelerator mass spectrometry (AMS) (Turteltaub et al., 1990). ^{14}C-labeled MeIQx was administered to mice at doses from 0.5 ng up to 5 mg/kg bw and DNA was isolated from liver tissue. AMS analysis clearly revealed a dose-dependent, linear increase of MeIQx DNA adduct levels from 500 ng up to 5 mg, whereas no differences were detected at very low dose levels ($\leq$5 ng/kg bw) due to technical limitations (Turteltaub et al., 1990). Subsequently, a

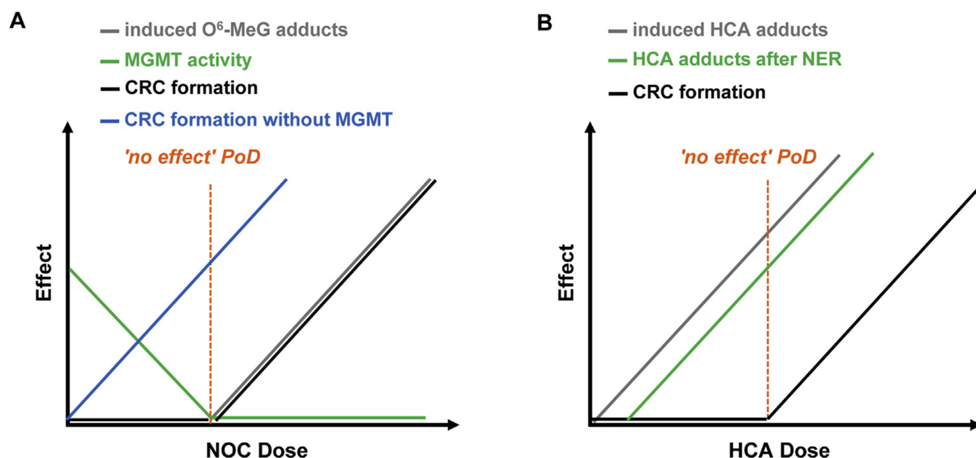

Fig. 5. Dose-response of NOC and HCA-induced DNA damage and colorectal carcinogenicity. A At low doses of NOC, O^6-MeG adducts are completely repaired by MGMT, which gets inactivated and is degraded (green line). If the cellular MGMT pool is depleted at higher NOC doses, O^6-MeG adducts (grey line) increase in a linear fashion. As O^6-MeG is the principal mutagenic and carcinogenic lesion induced by NOC, colorectal tumor formation increases accordingly after MGMT depletion (black line). Thus, the 'no effect' PoD for a genotoxic and carcinogenic threshold depends on the cellular MGMT level. Given that MGMT is lacking (*e.g.* due to pharmacological inhibition, epigenetic silencing or genetic ablation), the dose-response is converted from a non-linear to a linear relationship (blue line). **B** HCA such as PhIP induce dG-C8-PhIP adducts in a linear, dose-dependent manner (grey line). At very low doses, a no-observed-effect-level might exist for HCA adducts due to NER, which is saturated at higher doses (green line). Colorectal carcinogenicity (black line) exhibits a non-linear dose-response with a 'no effect' PoD, *i.e.* a carcinogenic threshold. The biological processes underlying this non-linear dose-response are yet to be elucidated and might involve NER, cell death or related mechanisms.

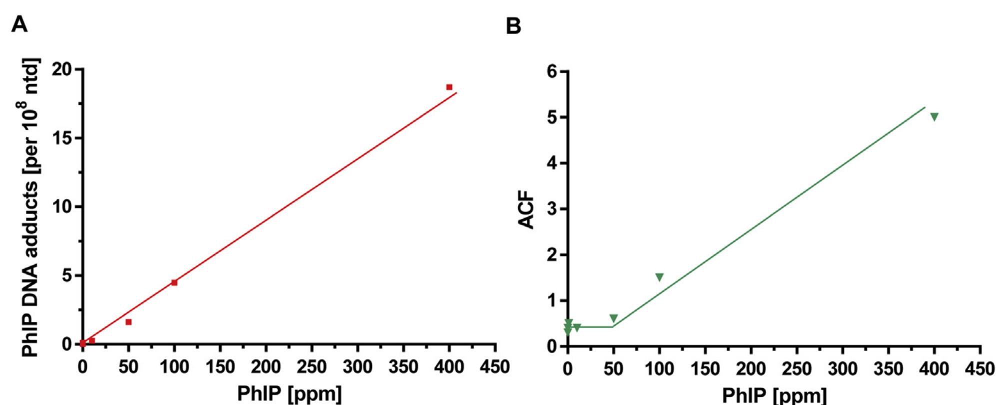

Fig. 6. Dose-response of PhIP-induced DNA adducts and ACF formation. A PhIP induces DNA adducts in a dose-dependent, linear manner without a genotoxic threshold. **B** The formation of ACF, a pre-neoplastic lesion resulting in CRC, exhibits a non-linear dose-response with an estimated carcinogenic threshold of about 50 ppm. Data was extracted from (Fukushima et al., 2004).

dose-response study was performed in rats that received IQ (0.01–20 mg/kg bw) by oral gavage. Both C8-IQ-dG and *N*2-IQ-dG adduct levels were dose-dependently augmented and displayed linearity as shown by the ^{32}P-postlabeling assay in hepatic DNA (Turesky et al., 1997). A more recent study addressed the dose-dependence of PhIP-induced DNA damage in the colon and its association with the occurrence of aberrant crypt foci (ACF), which are pre-neoplastic lesions (Fukushima et al., 2004). Rats were fed with a diet containing 0–400 ppm PhIP for 4 weeks and adduct levels were determined in colonic DNA by ^{32}P-postlabeling, revealing a linear relationship between 0.01 ppm up to 400 ppm, however without an increase at the lowest dose of 0.001 ppm PhIP (Fig. 6A) (Fukushima et al., 2004). Furthermore, the number of ACF was assessed following PhIP exposure for 16 weeks. Intriguingly, induction of ACF only occurred at doses ≥50 ppm, whereas no ACF formation was observed at doses of 10 ppm and below, pointing to the existence of a 'no effect' PoD for the carcinogenicity of PhIP in the large intestine, as derived from linear data presentation (Fig. 6B). In support of this notion, a study in rats exposed to MeIQx and the tumor promoter phenobarbital reported a lack of induction of pre-neoplastic GST-P positive foci in the liver at doses below 10 ppm, however without determining the MeIQx-DNA adducts (Fukushima et al., 2003). Very recently, a comparable study was conducted with both IQ and MeIQx in the absence of phenobarbital, which also showed no increase in the number of GST-P positive liver foci at doses below 10 ppm (Fukushima et al., 2016). It should be noted that hepatic HCA-DNA adducts increased in a dose-dependent manner from 0.01 up to 100 ppm (Fukushima et al., 2016). Further evidence for a no-observed-effect-level upon chronic PhIP exposure stems from a work, in which rats were injected twice with the tumor initiator AOM followed by a diet with increasing PhIP doses (0–200 ppm) over 36 weeks (Doi et al., 2005). PhIP doses up to 10 ppm did not increase the background levels of AOM-induced colonic tumors, whereas high doses ≥50 ppm strongly elevated tumor incidence and multiplicity for both colorectal adenomas and adenocarcinomas (Doi et al., 2005). Altogether, PoDs have been derived for the liver and colon carcinogenicity of HCAs using neoplastic precursor lesions (ACF, GST-P foci) as biological endpoint (Fig. 5B). Interestingly, all tested AIAs were genotoxic at levels below the determined 'no effect' PoD for carcinogenicity, suggesting that downstream events such as DNA repair or elimination of damaged or initiated cells by cell death may account for the observed non-linearity in hepato- and colon carcinogenesis as discussed elsewhere (Thomas et al., 2015). In this regard, it would be very interesting to investigate how deficiency in NER will impact the dose-response of HCA-induced liver and colon cancer (see Fig. 5B).

5. Concluding remarks

DNA repair is at the forefront in protecting our genome against DNA-damaging compounds, which are found in the diet, but can also arise endogenously in the gastrointestinal tract. With regard to CRC, the food-borne and endogenously generated NOC and HCA are particularly important as carcinogenic insults. MGMT was revealed as a major defense against NOC-induced DNA damage and colorectal cancer formation. It is reasonable to posit that dietary intake and/or *in situ* production of NOC below the tissue capacity of MGMT-mediated repair will be safe, whereas daily consumption of high levels of processed and red meat will overwhelm MGMT activity in the liver and large bowel, resulting in the initiation of tumor cells due to persistent O^6-MeG lesions. To study this hypothesis in more detail, robust and sensitive methods are required to assess and monitor the levels of O^6-MeG and other O^6-alkylguanine adducts in liver and colon tissue, *e.g.* obtained during routine colonoscopy. The mechanisms underlying the observed non-linear dose-response in HCA-induced colorectal carcinogenesis in mouse models remain to be determined. The discrepancy between linear DNA adduction and non-linear ACF formation might be attributable to lower mutagenicity or slower repair rates, which could be addressed using mouse models defective in specific functions involved in NER. Other related pathways such as cell death and senescence may also contribute to the non-linearity by eliminating initiated cells or halting cell cycle progression. It is conceivable that comparable dose-response relationships exist for red meat/heme-mediated CRC formation. This is an extremely important and complex issue as it involves various genotoxic insults and endogenous processes, very likely also the microbiome, as well as inflammation-related tumor promotion.

Conflict of interest

none.

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