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GENOTOXICITY AND CARCINOGENICITY

The *Brassica*-derived phytochemical indolo[3,2-*b*]carbazole protects against oxidative DNA damage by aryl hydrocarbon receptor activation

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Abstract Epidemiological studies suggest that a high intake of *Brassica* vegetables protects against colon carcinogenesis. *Brassica* vegetables are rich in glucosinolates which are hydrolysed during digestion to various products including indole-3-carbinol. In animal studies, a protective effect of indole-3-carbinol has been demonstrated in colon carcinogenesis. Indole-3-carbinol is highly unstable and, therefore, the observed protection likely results from condensation products of indole-3-carbinol, e.g. diindolylmethane or indolo[3,2-*b*]carbazole (ICZ). Interestingly, ICZ is a potent activator of the aryl hydrocarbon receptor (AhR), a transcription factor known to mediate toxic effects of environmental pollutants, such as dioxin and polycyclic aromatic hydrocarbons. Here, we show that ICZ protects against oxidative DNA damage in various cell lines including the colon carcinoma cell line Caco-2. When preincubated for 24 h, ICZ decreases DNA single-strand break (SSB) and 8-oxo-dG formation induced by tertiary-butylhydroperoxide (t-BOOH), hydrogen peroxide or benzo[*a*]pyrene. Simultaneous addition of ICZ does not protect against t-BOOH-induced SSB formation, which disproves a direct radical scavenging effect. The repair of SSBs was not enhanced, but the data indicate that ICZ attenuates the ROS level following t-BOOH. The antioxidant response factor Nrf2 was not activated following ICZ. Functional inhibition of the AhR and AhR-/ARNT-defective cell lines demonstrate that the AhR/ARNT pathway is mandatory for the observed ROS defence caused by ICZ, supporting the hypothesis that AhR-mediated regulation of defence genes is involved. The data point to a hitherto unknown protective function of ICZ and a novel role of the AhR in the defence against oxidative DNA damage.

Keywords Aryl hydrocarbon receptor · Indolo[3,2-*b*]carbazole · Oxidative DNA damage · Protection

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Introduction

Colorectal cancer is the third common type of cancer worldwide. Epidemiological studies suggest that a high intake of vegetables and fruits protects against carcinogenesis in various organs including colon. Especially vegetables of the Brassicacea family seem to be protective against colon cancer (Verhoeven et al. 1997; Higdon et al. 2007; Traka and Mithen 2009). Although the underlying mechanisms are not fully understood, the cancer chemopreventive effects of *Brassica* vegetables are attributed to glucosinolates. To date, more than 100 glucosinolates have been identified. For instance, broccoli and Brussels sprouts contain high amounts of glucobrassicin, a 3-indolylmethyl-substituted compound. It is generally assumed that the protective effects of the glucosinolates are not mediated by the compounds themselves, but rather by their hydrolysis products. Upon cellular damage, typically upon mastication, the plant-derived enzyme myrosinase is released, which leads to metabolisation of the glucosinolates to isothiocyanates, thiocyanates and indoles among other products (Holst and Williamson 2004, Fig. 1). For instance, indole-3-carbinol (I3C) is a major autolysis product derived from glucobrassicin, which has gained broad attention due to its

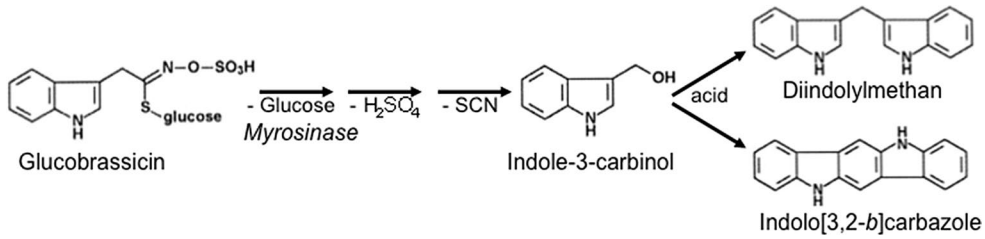

Fig. 1 Formation of ICZ. Physical damage of the plant material, e.g. broccoli, results in the release of glucosinolates and myrosinase (β-thio-glucosidase) from different cell organelles, which leads to hydrolysis of the glucosinolates by the myrosinase and further metabolism. For instance, glucobrassicin is hydrolysed with subsequent release of sulphate to the unstable indolylmethyl-isothiocyanate, which releases the thiocyanate group, thereby forming indole-3-carbinol. Under acid condition, indole-3-carbinol dimerises to various metabolites, among them ICZ (according to Holst and Williamson 2004)

modulating effects on carcinogenesis. When administered after exposure to a carcinogen, I3C acts as a tumour promoter and enhances carcinogenesis (Birt et al. 1986; Bailey et al. 1987; Yoshida et al. 2004). It has also been reported that I3C itself leads to DNA adduct formation (Arif et al. 2000; Reddy et al. 2002). Importantly, when given before carcinogenic treatment, I3C reduces the formation of DNA adducts in vitro and in vivo (Shertzer 1984; Salbe and Bjeldanes 1989; Arif et al. 2000; He et al. 2000). In line with these observations, I3C inhibits carcinogen-induced tumour formation in several organs including colon (Wattenberg and Loub 1978; Stoner et al. 2002; Kassie et al. 2007). For instance, I3C reduces the formation of azoxymethane-induced aberrant colon crypt foci in rats (Stoner et al. 2002). I3C also inhibits colon carcinogenesis in the *APC*$^{Min/+}$ mouse (Kawajiri et al. 2009), which carries a mutation in the *APC* gene that leads to deregulation of β-catenin degradation. Of note, I3C is unstable at acidic pH, as it is found in the stomach, where it is further converted to various condensation products in vivo and in vitro, such as 3,3′-diindolylmethane (DIM), 2-(indol-3-ylmethyl)-3,3′-diindolylmethane (LTr-1) and indolo[3,2-*b*]carbazole (ICZ) (Bjeldanes et al. 1991; De Kruif et al. 1991; for review, see Nguyen and Bradfield 2008). Hence, the anti-carcinogenic effects of I3C are probably not mediated by the compound itself. While the protective role of DIM in carcinogenesis has been widely studied (Maruthanila et al. 2014), much less is known about the molecular effects caused by ICZ and its possible protective activities. Of note, in view of a potential genotoxic risk of I3C the identification of less genotoxic compounds with similar protective activity would be of great importance.

Interestingly, both ICZ and DIM are potent ligands of the aryl hydrocarbon receptor (AhR) (Bjeldanes et al. 1991; for review, see Nguyen and Bradfield 2008). The AhR is a transcription factor belonging to the basic helix-loop-helix/PER-ARNT-SIM family (Marlowe and Puga 2005). In the cytosol, the unliganded receptor forms a complex with two heat-shock proteins 90, the immunophilin homologous AhR-interacting protein (AIP, also known as ARA9 or XAP2) and the co-chaperone p23. Binding of the ligand leads to disruption of the complex and nuclear translocation of the AhR. After heterodimerisation with aryl hydrocarbon receptor nuclear translocator (ARNT), the AhR/ARNT heterodimer binds to specific enhancer sequences, known as xenobiotic-responsive elements (XREs) or dioxin-responsive elements (DREs), leading to transactivation of several genes encoding phases I and II xenobiotic-metabolising enzymes, such as *cytochrome P450 monooxygenases* (*CYP1A1*, *CYP1A2*, *CYP1B1*) and *glutathione-S-transferases*, *NADPH/quinone oxidoreductase* and *aldehyde dehydrogenase 3*, respectively (for review, see Nebert et al. 2004; Barouki et al. 2012). Although mechanistically not fully understood, it is generally accepted that the AhR mediates the toxic responses of environmental pollutants, such as polycyclic aromatic hydrocarbons, dioxins with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as prototype and polychlorinated biphenyls (PCBs) (Swanson and Bradfield 1993).

However, novel non-canonical AhR-driven pathways have been identified, and there is strong evidence for AhR functions beyond xenobiotic metabolism (Barouki et al. 2007; Tuomisto 2005; Dietrich and Kaina 2010). Noteworthy, the AhR plays a protective role in colon carcinogenesis in the *APC*$^{Min/+}$ mouse (Kawajiri et al. 2009). However, it is not known to which extent the protective properties of I3C (and its derivatives) can be attributed to AhR activation in carcinogen-induced tumour formation.

It has been shown that administration of ICZ significantly reduces benzo[*a*]pyrene (B[*a*]P)-induced DNA adduct formation in the human colon carcinoma cell line Caco-2. The protection was only observed at very low B[*a*]P concentrations, and the mechanism is still unknown (De Waard et al. 2008). In the colon carcinoma cell line LS-174, pretreatment with sulforaphane, a potent inducer of the antioxidant transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), together with ICZ was shown to reduce the level of DNA single-strand breaks (SSBs) in response to B[*a*]P or hydrogen

peroxide (H_2O_2) (Bonnesen et al. 2001). These data suggest a possible DNA-protecting effect of ICZ. In the present work, we show that ICZ indeed protects against oxidative DNA damage in a variety of cell lines including Caco-2. We further demonstrate that activation of the AhR and ARNT is required for ICZ-mediated protection and that Nrf2 is not involved. Hence, we have identified a hitherto unknown defence function of the AhR directed against oxidative DNA damage.

Materials and methods

Cell culture

The human colon epithelial cell line Caco-2 derived from a colorectal adenocarcinoma was obtained from Cell Line Service (CLS) (Heidelberg, Germany), and the mouse hepatoma cell lines Hepa1c1c7, Hepa-c4 (lacking functional ARNT protein due to a point mutation in the ARNT gene) and Hepa-c12 (expressing reduced levels of AhR mRNA and protein) were purchased from the American Type Culture Collection (Rockville, MD, USA). The human keratinocyte cell line HaCaT (Boukamp et al. 1988) was a kind gift by Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany). V79 cells were previously described (Roos et al. 2009). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA), supplemented with 1 % non-essential amino acids, 2 mM glutamine, penicillin and streptomycin (each 100 U/ml), and 10 % fetal calf serum (FCS) (Sigma, St. Louis, MO, USA). The Hepa cell lines and HaCaT cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA), supplemented with 2 mM glutamine, penicillin and streptomycin (each 100 U/ml), and 10 % FCS. V79 cells were cultured in DMEM/F12 medium supplied with 5 % fetal bovine serum. Cells were kept at 37 °C in a humidified atmosphere containing 5 % CO_2.

Determination of cell number

Caco-2 cells were seeded at a concentration of 9×10^3 cells/cm^2 and treated with ICZ (synthesised by Albrecht Seidel, Biochemical Institute for Environmental Carcinogens, Grosshansdorf, Germany) after adherence of the cells to the dish (approximately 4 h). Cells were harvested after 24 and 48 h. For determination of cell number, cells were washed, trypsinised, and counted in a hemocytometer. The viability was checked by trypan blue exclusion.

Western blotting

Cells were lysed in hot Laemmli sample buffer (Laemmli 1970) or nuclear extracts were obtained according to Weiss et al. (2008). Protein concentration was determined according to Smith et al. (1985). Equal amounts of protein (20–35 μg per lane) were separated by SDS-PAGE (7.5–12.5 %) and electroblotted onto Immobilon membranes (Merck Millipore, Darmstadt, Germany). The blots were blocked for 1 h with 5 % low-fat milk powder in TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 0.1 % Tween 20 and then incubated for 1.5 h at room temperature with anti-CYP1A1 (1:1000, Santa Cruz, CA, USA), anti-OGG1 (1:500, GeneTex, Irvin, CA, USA), anti-APE (1:2000, Novus Biologicals, Littleton, CO, USA), anti-PARP1 (1:600, BD Transduction Laboratories, San Jose, NJ, USA), anti-XRCC1 (1:1000, GeneTex), or anti-Nrf2-antibody (1 μg/ml, R&D Systems, Minneapolis, MN, USA) followed by incubation with horseradish-peroxidase-conjugated secondary antibody and ECL detection (Cell Signaling, Beverly, MA, USA) according to the manufacturer's instructions. To control for equal loading, the blots were stripped and reprobed with anti-p38, anti-HSP90 or anti-ERK2 antibody (each 1:1000, Santa Cruz) followed by ECL detection as described above or by detection with alkaline phosphatase, respectively.

Colony formation assay

Caco-2 cells ($n = 400$) were seeded in 60-mm Petri dishes. After attachment, cells were exposed to ICZ for 24 h. The medium was changed and after 7–10 days, colonies were fixed with methanol, stained with Giemsa/crystalviolet solution and counted.

HPRT assay

Forward mutations at the *Hprt* locus leading to resistance to 6-thioguanine in V79 cells were used to analyse a potential mutagenic effect of ICZ. The assay was performed according to Glatt et al. (1998). Briefly, 10^4/cm^2 V79 cells were seeded onto 10-cm dishes in triplicates for each treatment. After 18 h, cells were incubated for 2 h with ICZ or DMSO at the indicated concentrations. MNNG was used as a positive control. Three days later, cells were trypsinised and counted to determine toxicity of the compounds. The cells were subcultured in normal medium for another 3 days and then subcultured again in the presence of 6-thioguanine (6.7×10^3 cells/cm^2, 10 cm dishes, 14 dishes) and, in parallel, in normal medium to assess cloning efficiency (250 cells/60 mm dish, 3 dishes). After 10 days, the cultures were fixed and stained, the colonies were counted, and mutant frequency was determined.

Quantification of γH2AX foci

V79 cells (2×10^4/well) were seeded onto precleaned sterile cover slips in six-well culture plates. Two days later,

they were treated for 2 h with the tested concentrations of ICZ or the DMSO solvent (0.3 %). After treatment, the medium was changed and cells were further incubated for 6 or 24 h. Immunostaining for γH2AX was performed as previously described (Nikolova et al. 2014). Briefly, the cells grown on cover slips were washed in phosphate-buffered saline (PBS), fixed with 4 % paraformaldehyde for 15 min at room temperature and further post-fixed with 1–2 ml ice-cold methanol for 10 min at −20 °C. After washing/rehydration steps with PBS, the blocking reagent (PBS, 0.25 % Triton X-100, 10 % goat serum) was added for 1 h. The primary antibody (mouse anti-phospho-H2AX-Ser139, Millipore, Darmstadt, Germany) was diluted to 1:1000 in PBS/0.25 % Triton X-100 buffer, added to the cells and left overnight at 4 °C. The next day, the cells were washed three times in PBS and the secondary antibody was added (Alexa488 Fluor $F(ab')_2$ fragment goat anti-mouse, Life Technologies, CA, USA), diluted to 1:500 in the same buffer. The samples were stored in the dark at room temperature for 1 h. After the washing process, 10 µl antifade medium (Vectashield, Vector Laboratories, CA, USA) with DAPI was dropped onto clean slides and the cover slips were transferred onto the slides and fixed with nail polish. For each treatment level, 500 cells were analysed in each experiment. All experiments were done at least three times. Microscopic images were screened and captured using Zeiss Axio Imager M1 (Carl Zeiss) supplied with the Metafer4 Software (MetaSystems, Altlussheim, Germany). The image galleries were further processed and the quantification performed using ImageJ (Fiji) with suitable batch-macro as previously described (Nikolova et al. 2014).

Detection of 8-oxo-dG

Caco-2 cells were seeded on glass coverslips, cultured and treated with ICZ and H_2O_2 as described in the figure legend. Cells were fixed and permeabilised for 5 min with ice-cold methanol/acetone (2:1) under yellow light, air-dried and rehydrated by incubation with PBS for 10 min at room temperature. Digestion of RNA was performed by addition of RNAse A (0.4 µg) and RNAse T1 (0.25 U) for 1 h at 37 °C. After washing, cells were exposed to alkaline solution (60 % 70 mM NaOH/140 mM NaCl, 40 % methanol) for 5 min on ice. Cells were washed and proteolysis was performed by incubation with 0.1 % trypsin for 30 s at 37 °C. Cells were washed and exposed to proteinase K (2 µg/ml) in 20 mM Tris/HCl containing 20 mM $CaCl_2$, pH 7.5, for 10 min at 37 °C. After washing with PBS/0.2 % glycin, unspecific binding was blocked by incubation with 1 % casein/PBS (blocking buffer) for 30 min at room temperature. Anti-8-oxo-dG-antibody (Squarix, Marl, Germany) was added at a dilution of 1:100 in blocking buffer, and cells were incubated overnight at 4 °C. Cells were washed with 0.05 % Tween 20/PBS at room temperature and exposed to Cy3-conjugated anti-mouse secondary antibody (1:800, Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature in the dark. After washing, cells were exposed to To-Pro-3 (Thermo Fisher Scientific, MA, USA) for nuclear staining. Cells were washed with both methanol and PBS and finally mounted on glass slides in Vectashield mounting medium (Vector Laboratories, CA, USA). Cells were visualised by a LSM 710 (Zeiss, Oberkochen, Germany).

Alkaline and modified comet assay

Cells were seeded to semi-confluence and allowed to grow for 24 h. Cells were treated with ICZ, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Amchro, Hattersheim, Germany) or sulforaphane (Sigma, St. Louis, MO, USA) and were then exposed to t-BOOH (Sigma), H_2O_2 (Sigma) or B[*a*]P (kindly provided by Jan Vondracek and Miroslav Machala, Brno, Czech Republic) as described in the figure legends. In some experiments, cells were incubated with CH-223191 (Calbiochem, Darmstadt, Germany), 3′-methoxy-4′nitroflavone (MNF) (kindly provided by Josef Abel, Leibnitz Research Institute for Environmental Medicine, Düsseldorf, Germany) or trigonelline (Sigma) prior to ICZ treatment. The cells were harvested and subjected to an alkaline comet assay (Olive and Banath 2006). About 10^4 cells were mixed with 120 µl low melting agarose (0.5 %) and transferred onto slides precoated with agarose. Lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton X-100, pH 10) was performed (Caco-2 cells for 20 min, HaCaT for 60 min, Hepa cells for 50 min) at 4 °C. Cells were placed in an electrophoresis chamber and mounted in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH13) for 20 min at 4 °C to allow unwinding of the DNA. Electrophoresis was then performed at 25 V and 300 mA (Caco-2 and HaCaT cells for 15 min, Hepa cells for 20 min). Slides were neutralised three times for 5 min with 0.4 M Tris, pH 7.5, fixed for 5 min with 100 % ethanol, air-dried for 2 h and then stained with 50 µg/ml propidium iodide. Comets were analysed by fluorescence microscopy using an Olympus BX50 equipped with a ColorView camera (Olympus, Münster, Germany). At least 50 cells/slide were scored using the Comet IV software (Perceptive Instruments Ltd., Bury St Edmunds, UK). The Fpg-modified comet assay was performed accordingly with the following modifications: after cell lysis, the slides were washed with enzyme buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8) and covered with either 50 µl of enzyme buffer or formamidopyrimidine-DNA glycosylase (FPG) protein (kindly provided by Bernd Epe, University of Mainz, Germany) in enzyme buffer and incubated for

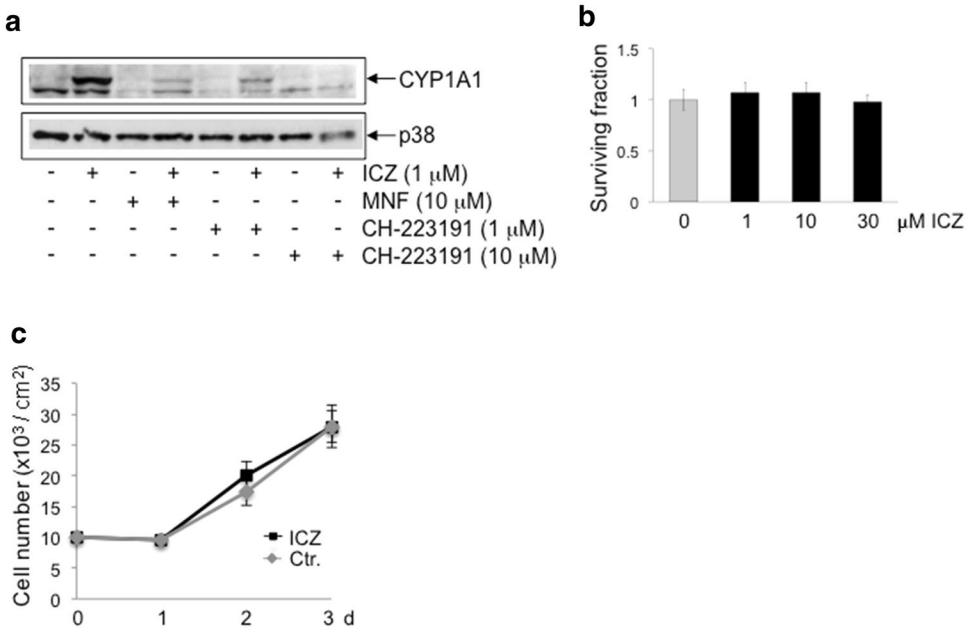

Fig. 2 ICZ is a potent, nontoxic AhR ligand. **a** AhR-dependent CYP1A1-induction in response to ICZ. Caco-2 cells were not treated or treated with ICZ (1 μM) for 24 h in the absence or presence of MNF or CH-223191 at the indicated concentrations. Western blot analysis was performed using an anti-CYP1A1-antibody. The blots were stripped and reprobed with an anti-p38-antibody to control equal loading. The blot shown is one representative out of two each leading to similar results. **b** Clonogenic survival in response to ICZ in Caco-2 cells. Cells were treated for 24 h with ICZ at the indicated concentrations. After 7–10 days, colonies were counted. The results represent the mean ± SD of three independent experiments. **c** Determination of cell proliferation in response to ICZ in Caco-2 cells. Cells were treated with ICZ after adherence of the cells to dish and cultured for 72 h

40 min at 37 °C. Thereafter, the protocol of the alkaline comet assay was followed by the DNA unwinding step as described above.

Antioxidative capacity

The antioxidative capacity was measured spectrophotometrically (Perkin Elmer Lambda 25 spectrometer) by analysing the decolorisation of the stable radical cation 2,2-diphenyl-1-picrylhydrazyl (DPPH) at 517 nm as described by Wätjen et al. (2007). Absorption was measured after 2 min of mixing the substances with the methanolic DPPH solution, and the final concentration of the sample in the cuvette was 76 μmol/L. The synthetic antioxidant TROLOX was used as a positive control.

Measurement of ROS

Cells were seeded, cultured and treated with ICZ as described in the figure legend. To determine intracellular ROS levels, cells were washed with PBS, covered with MEM without phenol red and incubated with the ROS probe CM-H_2DCFDA (Life Technologies, CA, USA) for 30 min at 37 °C. Cells were washed with PBS, covered with MEM without phenol red or FCS and treated with t-BOOH (30 μM) for 20 min. Cells were then washed with PBS, trypsinised and pelleted by centrifugation. Cells were resuspended in PBS, and flow cytometric analysis was performed by a FACSCalibur (BD Becton–Dickinson, Heidelberg, Germany).

Statistical analyses

Comparisons between treatments were made by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. A p value of <0.05 was considered to be significant.

Results

ICZ activates the AhR in a nontoxic dose range

ICZ has been described to be a potent AhR agonist (Bradfield and Bjeldanes 1987; Bjeldanes et al. 1991). We confirmed its AhR agonistic properties in human colon carcinoma Caco-2 cells. Western blot analysis revealed a strong induction of the prototypic AhR target gene CYP1A1 in response to ICZ (1 μM), which was blocked by co-administration of the AhR antagonist MNF (Lu et al. 1995; Zhou and Gasiewicz 2003) or by the AhR antagonist CH-223191 (Kim et al. 2006; Zhao

Table 1 HPRT gene mutation test of ICZ in V79 cells

Treatment	Mutants/10^6 cells, mean ± SD		
	Exp. 1	Exp. 2	Exp. 3
Control	5.4 ± 2.2 (5)	0.8 ± 0.7 (7)	6.4 ± 1.7 (5)
ICZ (1 μM)	5.2 ± 3.6 (5)	1.3 ± 0.9 (7)	2.6 ± 0.8 (5)
ICZ (10 μM)	n.d.	3.4 ± 2.7 (7)	6.4 ± 1.5 (5)
ICZ (30 μM)	3.8 ± 1.7 (5)	2.2 ± 1.8 (7)	2.4 ± 1.0 (5)
MNNG (1 μg/ml)	1629 ± 101 (5)***	599 ± 18 (7)***	1056 ± 76 (5)***

Cells were exposed to the test compounds for 2 h. After 70 h (first subcultivation), toxicity of the compounds in % of the corresponding negative controls was determined: 1 μM ICZ 108 ± 8.5 %, 10 μM ICZ 110 ± 8.8 %, 30 μM ICZ 96 ± 6.6 %, MNNG 44 ± 11 %

n.d. not determined

*** $p < 0.001$ (ANOVA followed by Bonferroni's multiple comparison test)

Table 2 γH2AX foci in response to ICZ in V79 cells

Treatment	γH2AX foci/cell, mean ± SD	
	6 h	24 h
Control	3.03 ± 0.83 (6)	4.16 ± 0.49 (6)
ICZ (1 μM)	2.83 ± 0.91 (3)	4.75 ± 0.72 (3)
ICZ (10 μM)	2.91 ± 1.25 (3)	4.51 ± 0.96 (3)
ICZ (30 μM)	2.85 ± 1.23 (3)	3.86 ± 1.05 (3)
MMS (1 mM)	19[a] (8 h)	

V79 cells were treated with ICZ for 2 h at the indicated concentrations. Medium was changed and immunostaining for γH2AX was performed after another 6 or 24 h. 500 cells per treatment were analysed by the Metafer 4 software

[a] Data from Nikolova et al. 2010

et al. 2010) (Fig. 2a). We therefore conclude that ICZ is a potent activator of the AhR. Since it has been shown that ICZ is cytotoxic in Caco-2 cells at high concentrations (>50 μM, Bonnesen et al. 2001), we analysed the cytotoxicity of ICZ at various concentrations using the sensitive colony formation assay. Figure 2b demonstrates that ICZ does not reduce the colony-forming ability of Caco-2 cells up to concentrations of 30 μM. In addition, proliferation of Caco-2 cells is not affected in the presence of 1 μM ICZ (Fig. 2c). We further investigated a potential genotoxic effect of ICZ by measuring mutagenicity and DNA double-strand break (DSB) formation. Mutagenicity of ICZ was examined by the HPRT assay in V79 cells. MNNG was used as a positive control. Only MNNG, but not ICZ, led to a significant induction of 6-thioguanine-resistant mutants (Table 1). Finally, we investigated in V79 cells the formation of γH2AX foci, which are markers for DNA DSBs and considered to be causal for chromosomal aberrations (Nikolova et al. 2010). We could not detect an accumulation of γH2AX foci after treatment with ICZ up to concentrations of 30 μM (Table 2). Hence, we conclude that ICZ is neither cytotoxic nor genotoxic up to concentrations of 30 μM.

ICZ protects against DNA strand break formation

We next investigated whether ICZ has an impact on the DNA reactivity of well-described genotoxic agents. To this end, Caco-2 cells were exposed to ICZ (1 μM) for 24 h and then exposed to the genotoxins t-BOOH (30 μM for 20 min), H_2O_2 (100 μM for 5 min) or B[*a*]P (1 μM for 24 h). All three agents produce oxidative stress (Epe et al. 1990; Park et al. 2009; and our own data not shown), which leads to DNA SSBs and oxidative DNA lesions, the most abundant being 8-oxo-dG. We used the alkaline comet assay, which detects SSBs, alkali-labile sites and DSBs. Figure 3a shows that pretreatment with ICZ for 24 h significantly protects against the induction of DNA strand breaks. Interestingly, pretreatment with ICZ for only 15 min followed by co-treatment with t-BOOH does not prevent t-BOOH-induced formation of DNA strand breaks (Fig. 3b), indicating that the protective effect of ICZ is not a result of direct ROS scavenging. We rather hypothesise that the protective effect of ICZ is related to alteration in gene expression triggered by the AhR. Of note, the protective effect of ICZ is not restricted to Caco-2 cells. It was also detected both in the human keratinocyte cell line HaCaT (Fig. 3c) and in the mouse hepatoma cell line Hepa1c1c7 (Fig. 3d). Interestingly, the protective effect of ICZ was as strong as the effect of the well-known Nrf2 activator sulforaphane (Kwak and Kensler 2010), as demonstrated in HaCaT (Fig. 3c) and Caco-2 cells (Fig. 3e).

ICZ impacts on oxidative DNA damage

The finding that ICZ pretreatment reduced the level of DNA strand breaks in cells exposed to t-BOOH, H_2O_2 or B[*a*]P led us to conclude that ICZ activates functions that protect against oxidative DNA lesions. We therefore performed a modified alkaline comet assay using the glycosylase Fpg, which is highly sensitive for detecting oxidative DNA lesions. Addition of the repair enzyme Fpg removes several oxidised bases from DNA, including 8-oxo-G, thereby producing apurinic sites, which are alkali labile. Since this assay is very sensitive, lower concentrations of the compounds were used. In Fig. 4a, we demonstrate that t-BOOH (3 μM), H_2O_2 (5 μM) as well as B[*a*]P (0.6 μM) induce Fpg-sensitive lesions, which are drastically reduced by 24 h preincubation with ICZ (1 μM). This strongly indicates that ICZ protects against oxidative DNA lesions. The data were confirmed by immunofluorescence studies using an anti-8-oxo-dG-antibody, which shows that 8-oxo-dG

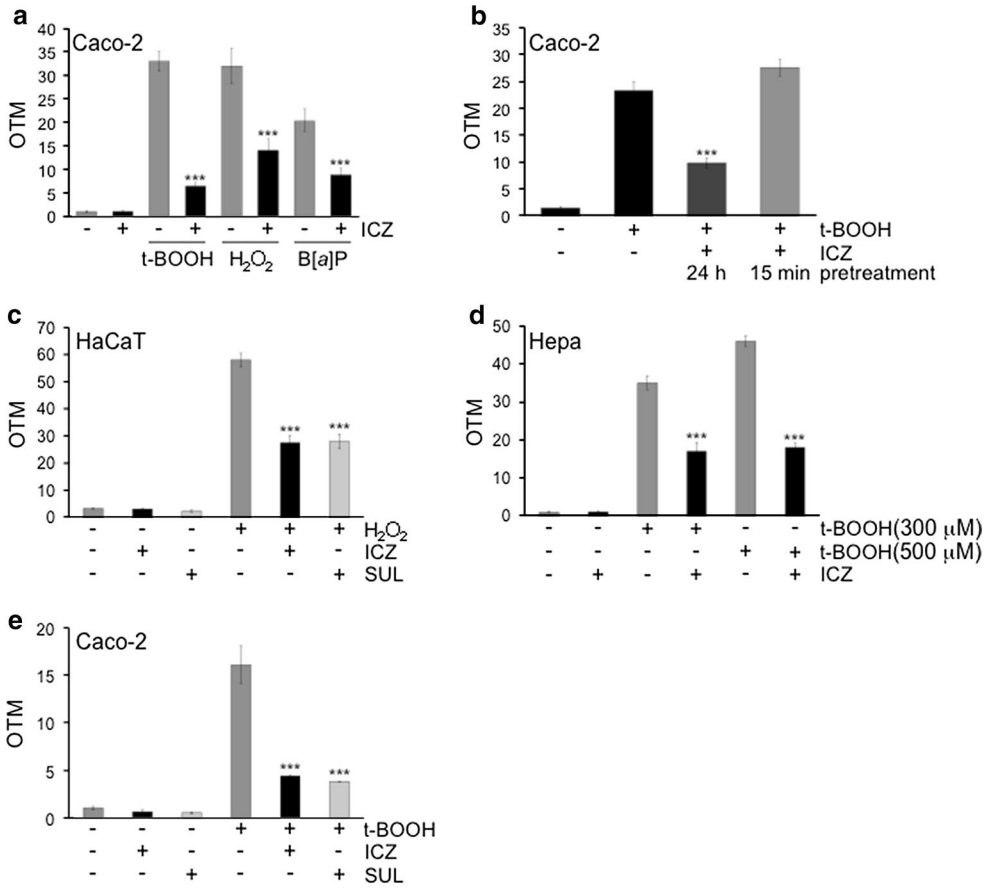

Fig. 3 ICZ protects against SSB formation in response to oxidative stress. **a–d** Alkaline comet assay detecting SSBs. Results are expressed as olive tail moments (OTM). **a** Caco-2 cells were treated for 24 h with ICZ (1 µM) and then exposed to t-BOOH (30 µM for 20 min at 37 °C), H_2O_2 (100 µM for 5 min on ice) or B[*a*]P (1 µM for 24 h at 37 °C). **b** Caco-2 cells were pretreated with ICZ either for 24 h or for 15 min and then exposed to t-BOOH (30 µM for 20 min at 37 °C). **c** HaCaT cells were pretreated with ICZ (1 µM) or sulforaphane (5 µM) and then exposed to H_2O_2 (30 µM for 5 min on ice). **d** Hepa cells were pretreated with ICZ (1 µM) for 24 h and then exposed to t-BOOH (20 min) at the indicated concentrations. The results represent the mean ± SD of three to five independent experiments, **e** Caco-2 cells were pretreated with ICZ (1 µM) or sulforaphane (5 µM) for 24 h and then exposed to t-BOOH (30 µM for 20 min at 37 °C). The results represent the mean ± SEM of one experiment out of two independent experiments each leading to similar results, ***$p < 0.001$ versus corresponding vehicle-treated cells

staining is reduced following H_2O_2 in cells pretreated with ICZ for 24 h (Fig. 4b).

ICZ does not improve the repair of oxidative DNA lesions

Next, we hypothesised that DNA repair, including SSB repair, might be improved by ICZ. We therefore investigated the repair of SSBs by the alkaline comet assay, which detects directly formed SSBs and SSBs formed during base-excision repair (BER). To this end, Caco-2 cells were pulse-treated with H_2O_2 and post-incubated for the indicated periods. Figure 5a shows that the initial SSB level is lower in the ICZ-pretreated cells, but the repair of SSBs appears not to be affected. Moreover, we could not find altered expression of the BER proteins OGG1, APE, PARP1 and XRCC1 in Caco-2 cells exposed to ICZ for 24 h (Fig. 5b). We conclude that ICZ has no impact on the SSB repair nor on the BER capacity of the cell.

ICZ reduces the intracellular ROS level

The low induced 8-oxo-dG level in ICZ-pretreated cells (Fig. 4) indicates that ICZ may activate ROS scavenging functions. To substantiate this, we investigated whether ICZ reduces the intracellular ROS level. Cells were pretreated or not with ICZ (24 h), loaded with the redox-sensitive probe CM-H_2DCFDA, exposed to t-BOOH (100 µM for 20 min) and analysed by flow cytometry. Pretreatment with ICZ strongly reduced t-BOOH-induced intracellular ROS levels (Fig. 5c). However, ICZ is not a radical scavenger *per se*, as confirmed by an in vitro DPPH assay (Fig. 5d).

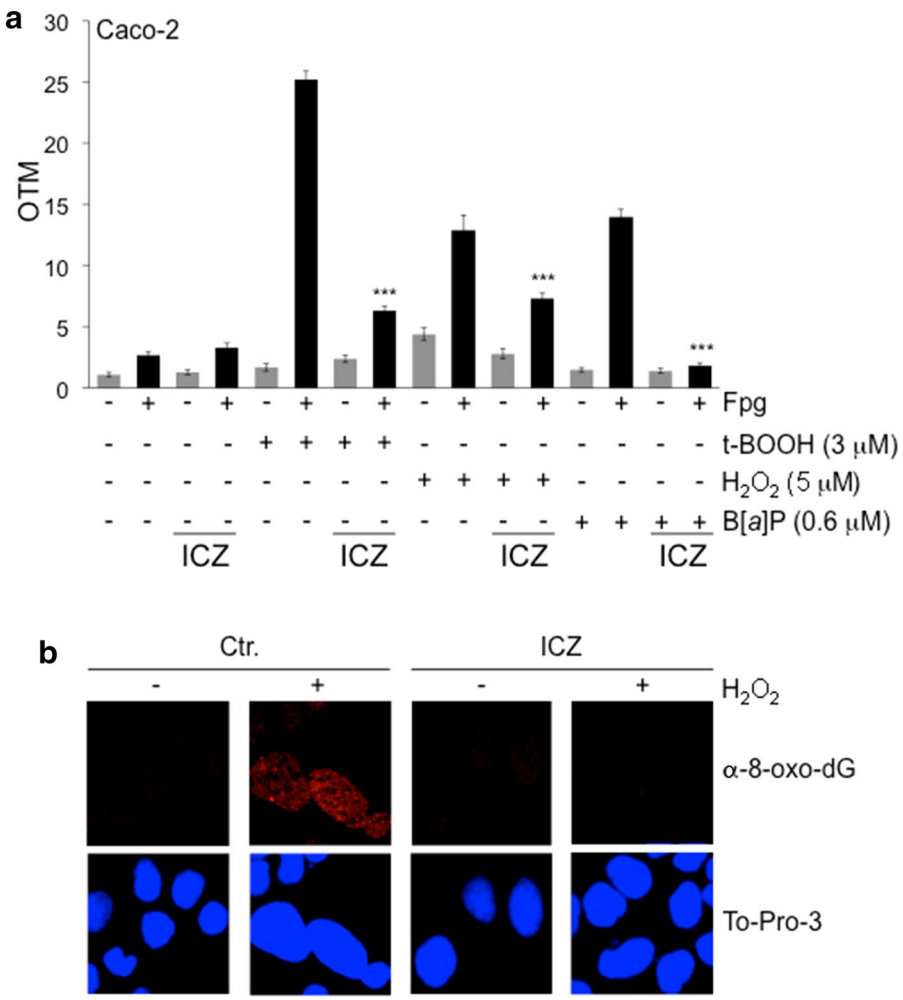

Fig. 4 ICZ protects against oxidative DNA damage. **a** Caco-2 cells were pretreated with ICZ for 24 h and exposed to t-BOOH (3 μM for 20 min at 37 °C), H_2O_2 (5 μM for 5 min on ice), or B[*a*]P (0.6 μM for 24 h at 37 °C). Oxidative DNA damage was determined by the Fpg-modified alkaline comet assay. Results are expressed as olive tail moments (OTM) and represent the mean ± SD of three independent experiments, ***$p < 0.001$ versus corresponding vehicle-treated cells. **b** Caco-2 cells were pretreated with ICZ (1 μM) for 24 h and then exposed to H_2O_2 (100 μM for 5 min on ice). Cells were stained with anti-8-oxo-dG antibody and To-Pro-3 to visualise the nuclei

Here, the direct antioxidant capacity of a compound can be measured by reduction in the absorbance (517 nm) of the radical DPPH. TROLOX, a synthetic vitamin E derivative, was used as a positive control (Fig. 5d).

ICZ does not activate the transcription factor Nrf2

Since our data point to ICZ-mediated induction of antioxidant functions, we focussed our interest on potential transcription factors that could be involved. The transcription factor Nrf2 is known to be a crucial mediator of the antioxidant response pathway. Under physiological conditions, the expression of Nrf2 is regulated by binding to Keap1, which mediates degradation of Nrf2. Upon oxidative stress, Nrf2 is stabilised by dissociation from Keap1 and translocated to the nucleus where Nrf2 dimerises with Maf or c-Jun and induces the expression of antioxidant enzymes such as γ-glutamylcysteine synthetase, thioredoxin and heme oxygenase-1 (for review, see Köhle and Bock 2006; Niture et al. 2014). To investigate whether ICZ activates the Nrf2 pathway, we analysed Nrf2 protein expression by Western blot analysis. While an incubation with the positive control TBHQ (tert-butylhydroquinone) resulted in an accumulation of Nrf2 protein, we could not detect any increase in Nrf2 protein in response to ICZ up to 6 h treatment (Fig. 6a). Similar results were obtained when cells were treated with ICZ for a period of 24 h (data not shown), indicating that Nrf2 is not involved. To confirm this notion, we tested whether the protective effect of ICZ on DNA strand break induction can be reversed by the Nrf2 inhibitor trigonelline (Arlt et al. 2013). In line with our data, the protective effect of ICZ was not inhibited by trigonelline (Fig. 6b). In control experiments, trigonelline blocked the TBHQ-induced nuclear translocation of Nrf2, as assessed by subcellular fractionation and Western blot analysis (Fig. 6c). The data led us to conclude that protection by ICZ against oxidative DNA damage is not a result of activation of Nrf2.

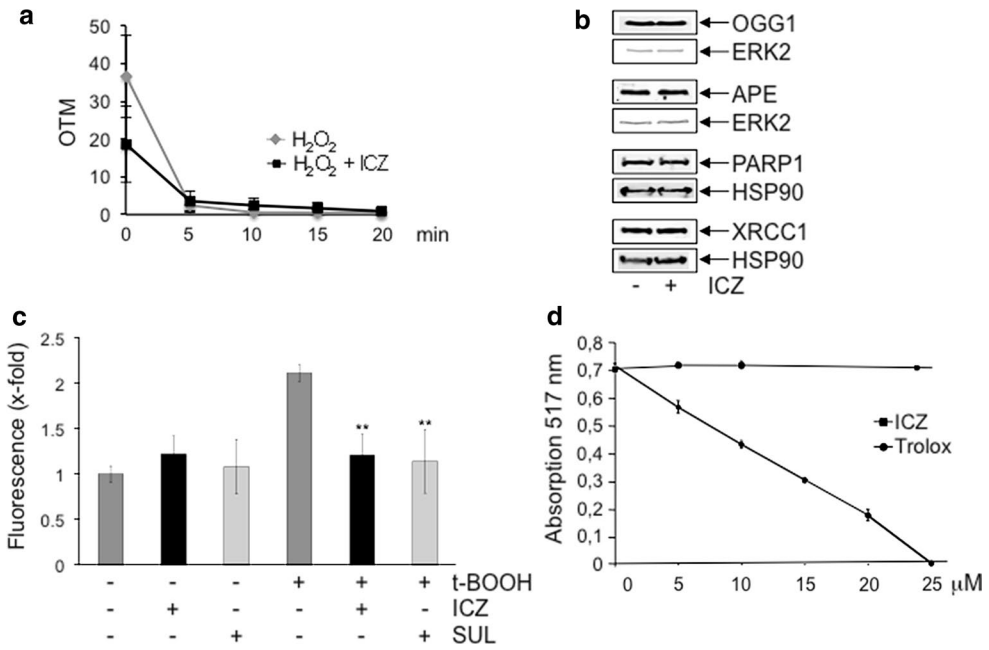

Fig. 5 ICZ decreases intracellular ROS formation. **a**, **b** DNA repair is not increased in response to ICZ. **a** Caco-2 cells were pretreated with ICZ (1 µM) for 24 h and then exposed to H_2O_2 (100 µM for 5 min on ice). SSBs were detected by the alkaline comet assay at the indicated time points after exposure. Results are expressed as mean ± SEM and represent one experiment out of two leading to similar results. **b** Caco-2 cells were treated with ICZ for 24 h. Protein expression of representative base-excision repair proteins was analysed by Western blot analysis using anti-OGG1, anti-APE, anti-PARP1 or anti-XRCC1 antibodies. The blots were stripped and reprobed with an anti-ERK2- or an anti-HSP90 antibody to control equal loading. The blots represent one out of two independent experiments each leading to similar results. **c** ICZ decreases intracellular ROS formation. Caco-2 cells were pretreated with ICZ (1 µM) or sulforaphane (5 µM) for 24 h and then exposed to t-BOOH (100 µM for 20 min). Intracellular ROS formation was detected by loading the cells with CM-H_2DCFDA and subsequent flow cytometry. Data are the mean ± SD of three independent experiments, $^{**}p < 0.01$ versus corresponding vehicle-treated cells. **d** ICZ is not a radical scavenger in vitro. Radical scavenging properties of ICZ were determined in vitro by the DPPH assay. Trolox was used as a positive control. A reduction in DPPH radical absorption indicates antioxidative capacity. Data are expressed as mean ± SD of three independent experiments

Protection by ICZ against oxidative DNA damage is mediated by the AhR

We finally addressed the question of whether the AhR is mandatory for the protective effect of ICZ. We first investigated the impact of the AhR inhibitor CH-223191 in an alkaline comet assay in Caco-2 cells. In the presence of CH-223191, the protective effect of ICZ on DNA strand break induction was completely reversed (Fig. 7a). Partial inhibition of the protective effect was also seen with the (partial) antagonist MNF (data not shown). In line with an involvement of the AhR, the prototypic AhR ligand TCDD also protected against t-BOOH-induced DNA strand break formation in Caco-2 cells (Fig. 7b). To confirm the causal role of the AhR in ICZ-provoked protection, we made use of Hepa-derived cell lines, which are deficient in the AhR (Hepa-c12) or ARNT (Hepa-c4). Whereas in the wild-type cell line Hepa1c1c7, ICZ significantly protects against t-BOOH-induced DNA strand break formation, the protective effect was completely vanished both in the AhR-deficient (Hepa-c12) and in the ARNT-deficient (Hepa-c4) mutants (Fig. 7c). We therefore conclude that the AhR/ARNT pathway is required for the protective effect elicited by ICZ on the induction of oxidative DNA lesions.

Discussion

Vegetable consumption, notably *Brassica* vegetables, is thought to be beneficial because of cancer-protective ingredients. One *Brassica*-derived compound is glucobrassicin, which generates several metabolisation products after ingestion, among them ICZ. The present work was aimed at investigating a possible protective effect of ICZ on oxidative DNA damage. Here, we show for the first time that ICZ protects against oxidative DNA damage. We proved its effect in several cell lines, including colon epithelial cells, which were treated with the model ROS generating compounds t-BOOH and H_2O_2. We also treated the cells with the environmental carcinogen B[*a*]P, which generates ROS as a by-product of drug metabolism (Park et al. 2009). We demonstrate that the AhR/ARNT complex is mandatory for

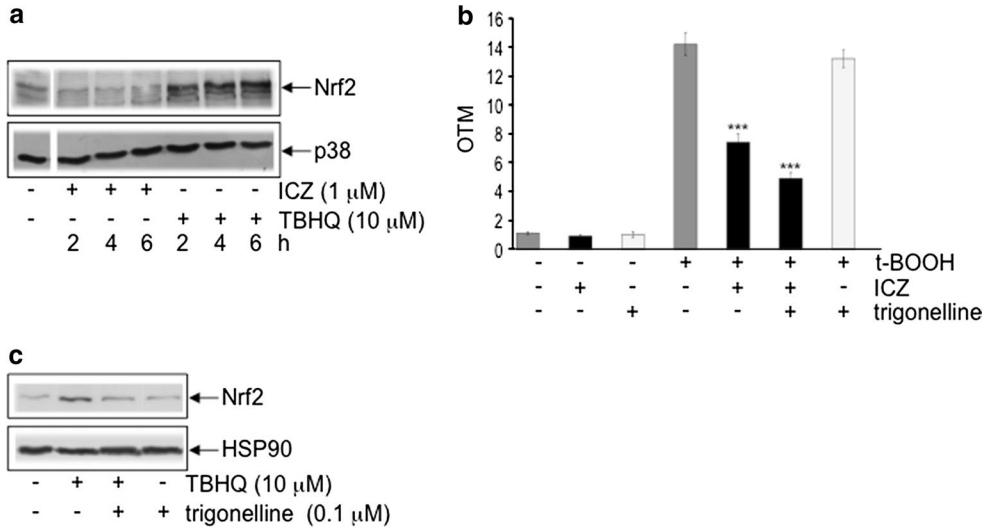

Fig. 6 ICZ does not activate the transcription factor Nrf2. **a** No increase in Nrf2 protein in response to ICZ. Caco-2 cells were treated with ICZ or TBHQ at the indicated concentrations for the indicated time periods. Western blot analysis of total cell extracts using an anti-Nrf2 antibody was performed to detect protein expression of Nrf2. The blots were stripped and reprobed with an anti-p38 antibody to control equal loading. The blot is representative of three independent experiments each leading to similar results. **b** ICZ-mediated protection is not reversed by trigonelline. Caco-2 cells were treated with ICZ for 24 h in the presence or absence of trigonelline (given 1 h prior to ICZ) and then exposed to t-BOOH (30 μM for 20 min). SSBs were detected as described in Fig. 3. Results are expressed as mean ± SEM and represent one experiment out of two independent experiments each leading to similar results. **c** Trigonelline prevents nuclear translocation of Nrf2. Caco-2 cells were pretreated with trigonelline for 1 h and then treated with TBHQ for 2 h. Nuclear extracts were subjected to Western blot analysis using an anti-Nrf2 antibody. The blots were stripped and reprobed with an anti-HSP90 antibody to control equal loading. The data are representative of two independent blots each leading to similar results

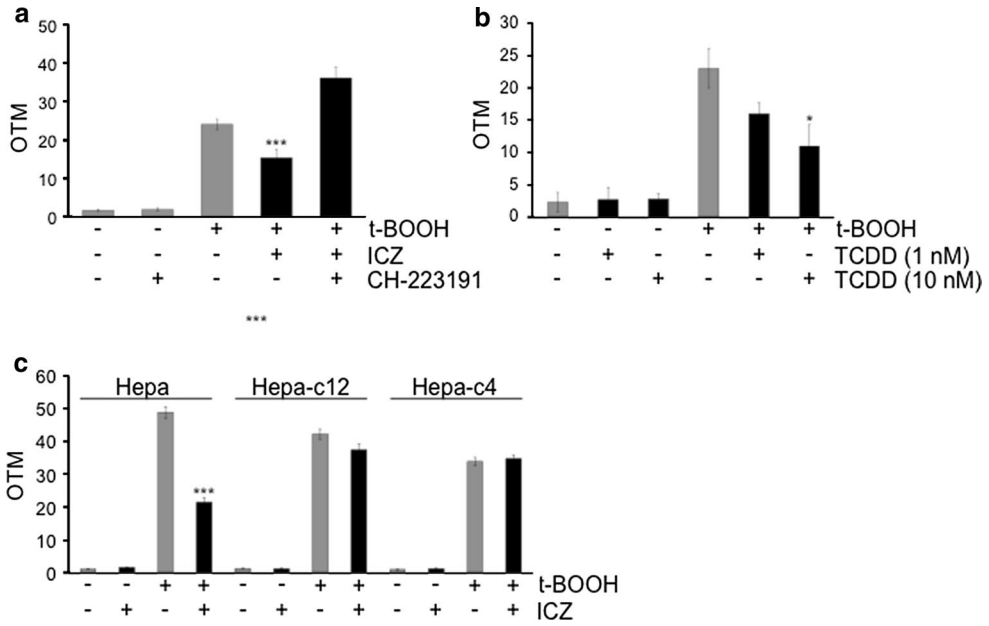

Fig. 7 The AhR is required for ICZ-mediated protection. **a**–**c** Detection of SSBs by alkaline comet assay. **a** Caco-2 cells were pretreated with ICZ (1 μM) for 24 h in the absence or presence of CH-223191 (given 1 h prior to ICZ) and then exposed to t-BOOH (30 μM for 20 min). **b** Caco-2 cells were pretreated with TCDD for 24 h at the indicated concentrations and then exposed to t-BOOH. **c** Hepa (wt), Hepa-c12 (AhR deficient), and Hepa-c4 (ARNT deficient) cells were pretreated with ICZ and then exposed to t-BOOH (300 μM for 20 min). Results are expressed as mean ± SD of three to five independent experiments, $*p < 0.05$; $***p < 0.001$ versus corresponding vehicle-treated cells

ICZ-mediated protection against oxidative DNA damage, whereas the antioxidant transcription factor Nrf2 is dispensable. We describe a novel function of the AhR, i.e. being involved in the regulation of defence functions directed against oxidative DNA damage.

ICZ is nontoxic in doses that protect against ROS-induced DNA damage

In line with previous reports (Bjeldanes et al. 1991; Kleman et al. 1994), ICZ acts as a potent AhR agonist in the colon epithelial cell line Caco-2. This was demonstrated by the increase in the prototypic AhR target gene CYP1A1 as well as by the inhibition of CYP1A1 induction by two different AhR antagonists, CH-223191 and MNF. This raises the question of a potential AhR-mediated toxicity of ICZ. Comparison of the acute toxicities of ICZ and TCDD in Long–Evans rats revealed that ICZ even at high doses did not induce any toxic effects in contrast to TCDD (Pohjanvirta et al. 2002). The fact that Long–Evans rats are about 100-fold more sensitive towards TCDD-mediated toxicity than humans, also argues against ICZ-mediated adverse health effects in humans. One plausible explanation lies in the shorter half-life of ICZ in comparison with TCDD (Chen et al. 1995).

ICZ showed neither an effect in the sensitive colony formation assay nor an influence on proliferation of Caco-2 cells. A study revealed induction of apoptosis following ICZ treatment in several colon cell lines including Caco-2 (Bonnesen et al. 2001). However, the concentrations were at least twofold higher than the highest concentration we used in our assays. We therefore conclude that ICZ does not induce apoptosis or another type of cell death in Caco-2 cells at concentrations below 30 µM. Such high concentrations of ICZ can hardly be achieved after *Brassica* consumption (Bjeldanes et al. 1991; Stresser et al. 1995; Kushad et al. 1999). The lack of toxicity in the colony formation assay argues against a DNA-damaging property of ICZ. In line, we could not detect any increase in DSB formation in response to ICZ up to a concentration of 30 µM. Moreover, no mutagenic effect could be seen in the HPRT assay. This is an important observation since DNA damage and mutagenicity have been shown for crude *Brassica* juices (Kassie et al. 1996) and some *Brassica*-derived compounds including I3C, its derivative 3-methyl-indole and neoglucobrassicin (Baasanjav-Gerber et al. 2011; Glatt et al. 2011; Schumacher et al. 2014; Wiesner et al. 2014). Of note, formation of DNA adducts has been detected in I3C-exposed rats (Arif et al. 2000), and I3C shows cytotoxicity in human breast cancer cells (Moiseeva et al. 2007). This is in line with our observation that I3C shows toxicity in the colony formation assay in Caco-2 cells (unpublished observation). Hence, in contrast to other *Brassica*-derived compounds, there is no indication so far for DNA-damaging or cytotoxic properties of ICZ. We cannot rule out that a metabolite of ICZ might be mutagenic since V79 cells, which we have used in our point mutation assays, are not metabolically competent. However, only mono- and dihydroxylated metabolites of ICZ have been identified so far, which are conjugated and excreted and hence not supposed to be mutagenic (Bergander et al. 2004). Thus, further studies are warranted to finally answer the question of the mutagenic potential of *Brassica* ingredients.

ICZ protects against oxidative DNA damage

ICZ protects against the formation of SSBs and of FPG-sensitive sites (both are markers for oxidative DNA damage) in response to H_2O_2 and t-BOOH, and the environmental carcinogen B[*a*]P, which is known to produce reactive oxygen species during its metabolism (Park et al. 2009; own unpublished data). The protective effect was not only restricted to Caco-2 cells, but also detected in human keratinocytes, murine hepatoma and also rat oval cells (unpublished observation). Of note, ICZ was reported to have only a marginal protective impact on SSBs after oxidative stress in the colon epithelial cell line LS-174, and protection was only seen when ICZ was combined with sulforaphane (Bonnesen et al. 2001). A possible explanation might rest on cell type specificity which is a typical feature of the AhR and hampers our understanding of AhR function (Denison et al. 2011).

ICZ leads to reduced intracellular ROS formation independent of Nrf2

The decrease in oxidative DNA lesions is very likely the result of ROS scavenging, which occurs not directly by ICZ, but rather by an unknown factor whose expression or protein level is regulated by ICZ. Thus, ICZ was able to reduce the ROS level as determined by flow cytometric analysis using the ROS-sensitive probe CM-H_2DCFDA. The mechanism of ROS scavenging following ICZ pretreatment is not clear. However, our observation that (i) simultaneous addition of ICZ and t-BOOH does not protect against oxidative DNA lesions and (ii) ICZ was ineffective in a cell free DPPH assay argues against a direct radical scavenging effect. Importantly, a longer preincubation period is required for eliciting protection, which points to ICZ-mediated alterations in gene expression. Since we ruled out a role of increased DNA repair, we hypothesised that ICZ provokes the expression of antioxidant enzyme(s), possibly by activation of Nrf2. Under normal conditions, Nrf2 is degraded by the proteasome system, which is regulated by the interaction with Keap1. Upon oxidative stress, Keap1 dissociates from the complex and hence Nrf2 protein is stabilised,

translocates to the nucleus, binds to antioxidant-responsive elements (ARE) and therefore induces expression of antioxidant enzymes (for review, see Köhle and Bock 2006; Niture et al. 2014). However, we could not detect any protein stabilization of Nrf2 in response to ICZ, nor could we reverse the protective effect of ICZ on SSB formation by the Nrf2 inhibitor trigonelline (Arlt et al. 2013). Our observations are in line with a previous report demonstrating that ICZ does not lead to activation of Nrf2-sensitive AREs in reporter assays or to expression of ARE-responsive genes in Caco-2 cells (Bonnesen et al. 2001). We therefore conclude that reduction in the intracellular ROS level and in oxidative DNA damage in ICZ-pretreated cells challenged with a ROS genotoxicant is independent of Nrf2.

The AhR/ARNT complex is required for ICZ-mediated protection against oxidative DNA damage

Three lines of evidence led us to conclude that the AhR/ARNT complex is key to the protective effect of ICZ on oxidative DNA damage: (i) pharmacological inhibition of the AhR prevents ICZ-mediated reduction in SSB formation, (ii) TCDD, a prototypic ligand of the AhR, also blocks t-BOOH-induced SSB formation, and (iii) ICZ-mediated protection is completely lost in AhR- or ARNT-deficient cells. These observations are in line with the hypothesis of an AhR-dependent pathway activated by ICZ. The question arises why B[*a*]P, a potent AhR ligand, then induces oxidative DNA damage. Firstly, we do not know whether the antioxidant pathway is stimulated after B[*a*]P-treatment. Although an overlap in gene expression is detected after exposure to TCDD or B[*a*]P, their gene expression profiles are not identical (Hockley et al. 2007). Secondly, we have not yet studied the kinetics of the antioxidant pathway. It is known that the B[*a*]P-metabolite B[*a*]P-7,8-dihydrodiol is metabolised by aldo–keto reductases forming B[*a*]P-7,8-diol. Sequential oxidation of the catechol group results in the formation of a semiquinone-radical and B[*a*]P-7,8-dione which is reduced again to B[*a*]P-7,8-diol by NADH-mediated mechanisms. This redox cycling of the B[*a*]P–metabolite B[*a*]P-7,8-diol leads to the formation of superoxide anions and H_2O_2 resulting in rapid induction of oxidative DNA damage (Park et al. 2009). It is possible that activation of the protective pathway occurs much more slowly.

In differentiated monolayers of Caco-2 cells, activation of the AhR by different AhR ligands, including ICZ, is known to induce the expression of breast cancer resistance protein (BCRP), which increases apical transport of phase II metabolites of B[*a*]P (Ebert et al. 2005, 2007). However, it is unlikely that an increase in BCRP expression is causal for ICZ-mediated protection against oxidative stress since (i) the expression of transporters is predominantly seen in differentiated monolayers, but not in exponentially growing cultures of Caco-2 cells (for review see Van Breemen and Li 2005), and (ii) an increase in BRCP expression has been shown to rather enhance sensitivity against oxidative stress (Krzyzanowski et al. 2014). We therefore hypothesise that ICZ induces the expression of antioxidant enzyme(s) which is in line with several other observations demonstrating antioxidant functions of the AhR. A classical XRE is found in the human superoxide dismutase 1 (SOD1) promoter (Cho et al. 2001; Park and Rho 2002), and the AhR seems to regulate expression of SOD1 and SOD2 (de Souza et al. 2011). We did not find any increase in SOD1 protein expression after ICZ exposure in Caco-2 cells (unpublished observation). In hepatoma cells, quercetin increases the expression of paraoxonase 1 (PON1) in an AhR-dependent manner (Gouédard et al. 2004), and dioxin-like PCBs result in elevation of PON1, 2 and 3 in mouse liver (Shen et al. 2015). In rat hepatoma cells, β-naphthoflavone treatment results in an increase in GSH expression and protection against nanoparticles-mediated ROS formation (Conolly et al. 2015). However, other possible target genes have to be considered since several non-canonical mechanisms and alternative binding regions for the AhR or the AhR/ARNT heterodimer have been identified (Sogawa et al. 2004; Boutros et al. 2004; Lo and Matthews 2012; Teino et al. 2012; Huang and Elferink 2012; Wilson et al. 2014). AhR-dependent, but DRE-independent regulation of gene expression of sulfiredoxin 1 has been reported recently (Sarill et al. 2015). Finally, transcription might be indirectly regulated by AhR-mediated upregulation of components of the transcription factor AP-1, such as c-Jun or JunD (Hoffer et al. 1992; Weiss et al. 2005, 2008). However, we could not detect any upregulation of c-Jun or JunD in response to ICZ in Caco-2 cells (unpublished observation). Alternatively, the AhR might induce post-translational protein modifications resulting in an increased protein level of an antioxidant enzyme. It is well known that the AhR also activates non-genomic pathways and interacts with several signalling pathways, such as Src, PKC and MAPK (Matsumura 2012; Puga et al. 2009). Hence, the downstream target(s) of the AhR/ARNT pathway mediating the protection against oxidative stress in Caco-2 cells still has to be identified.

Although our finding of a protective function of the AhR/ARNT pathway against oxidative DNA damage is in line with other observations describing antioxidant functions of the AhR as outlined above (de Souza et al. 2011; Sarill et al. 2015), it contrasts with the described oxidative stress in response to TCDD (for review, see Dalton et al. 2002; Stohs and Hassoun 2012). In vitro, production of ROS can be explained among other mechanisms by the induction of CYP1A1 (and CYP1B1), uncoupling of electron transfer and hence superoxide release (for review see Stohs and Hassoun 2012). Besides, CYP1A2 protects against

ROS formation by scavenging free electrons (Shertzer et al. 2004). Oxidative stress results from the net balance of oxidative and antioxidative mechanisms. In view of the well-known cell type and organ specificity of AhR function, it is plausible to assume that, depending on the cell type or organ, oxidative or antioxidative AhR pathways predominate. For instance, an increase in ROS production and 8-oxo-dG in the DNA after TCDD exposure has been observed in primary hepatocytes, but not in HepG2 cells (Knerr et al. 2006). To note, the observed effects on DNA damage in vitro are generally quite small (Knerr et al. 2006; Lin et al. 2007). Park and co-workers demonstrated an increase in 8-oxo-G in the medium of TCDD-treated Hepa1c1c7 cells, but they failed to detect 8-oxo-dG in the DNA of the cells which the authors explain by efficient DNA repair (Park et al. 1996). Interestingly, ICZ (5 μM) also induced release of 8-oxo-G in the medium albeit to a much lesser extent. This is consistent with our observation that neither ICZ nor TCDD produced DNA SSBs or 8-oxo-dG formation in the cells we tested, including Hepa1c1c7. Protective effects on DNA damage, as we describe here, keeping the level of oxidatively damaged DNA low, despite generation of oxidative stress, would also explain the lack of TCDD-mediated mutagenicity in rats (Thornton et al. 2001). However, we have not studied the effect of ICZ on mitochondrial ROS production and mitochondrial DNA damage which significantly contribute to oxidative stress (Shen et al. 2005). In vivo, additional factors may contribute to generation of ROS after TCDD treatment, at least in rodent liver. In female rats, oxidative DNA damage in liver is entirely dependent on estrogens (Tritscher et al. 1996; Wyde et al. 2001). In addition, TCDD induces pronounced liver inflammation including infiltration of macrophages (for review, see Stohs and Hassoun 2012). As stated above, ICZ is not toxic to rat liver, and hence, it is very unlikely that ICZ provokes a similar inflammatory response. Oxidative stress in the colon of rodents in response to TCDD has not been detected so far. In line with the observed anti-inflammatory function of the AhR in DSS-induced colitis in mice (Ji et al. 2015), it is unlikely that ICZ induces oxidative stress in the colon. However, in vivo studies are required to finally answer this question.

Conclusions

Oxidative stress is involved in the pathogenesis of various colorectal diseases, such as Crohn's disease, ulcerative colitis and colorectal cancer (Klaunig and Kamendulis 2004; Klaunig et al. 2010; Almenier et al. 2012). It induces a plethora of DNA damages with 8-oxo-dG being not only the most abundant (Dizdaroglu et al. 2002), but also a mutagenic lesion. Downregulation of intracellular ROS was shown to protect against colorectal carcinogenesis (Yang et al. 2014; for review, see Saw and Kong 2011). Glucosinolate-rich diet attenuates colon carcinogenesis in mice, probably by upregulation of Nrf2 and some of its target genes (Lippmann et al. 2014). Hence, downregulation of oxidative stress by activating defence mechanisms seems to be promising in reducing colon cancer (at least in animal models). Here, we describe a novel AhR-dependent pathway for the protection against ROS-induced DNA damage, which is independent of Nrf2. Furthermore, we present a novel protective role of ICZ whose molecular elements remain to be investigated.

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