Xanthohumol Prevents DNA Damage by Dietary Carcinogens: Results of a Human Intervention Trial

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Abstract

Xanthohumol (XN) is a hop flavonoid contained in beers and soft drinks. In vitro and animal studies indicated that XN has DNA and cancer protective properties. To find out if it causes DNA protective effects in humans, an intervention trial was conducted in which the participants (n = 22) consumed a XN containing drink (12 mg XN/P/d). We monitored prevention of DNA damage induced by representatives of major groups of dietary carcinogens [i.e., nitrosodimethylamine (NDMA), benzo(a)pyrene (B[a]P) and the heterocyclic aromatic amine 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)]. Lymphocytes were collected before, during, and after the intervention and incubated with the carcinogens and with human liver homogenate (S9). We found substantial reduction of B(a)P and IQ (P < 0.001 for both substances) induced DNA damage after consumption of the beverage; also, with the nitrosamine a moderate, but significant protective effect was found. The results of a follow-up trial (n = 10) with XN pills showed that the effects are caused by the flavonoid and were confirmed in γH2AX experiments. To elucidate the underlying mechanisms we measured several parameters of glutathione related detoxification. We found clear induction of α-GST (by 42.8%, P < 0.05), but no alteration of π-GST. This observation provides a partial explanation for the DNA protective effects and indicates that the flavonoid also protects against other carcinogens that are detoxified by α-GST. Taken together, our findings support the assumption that XN has anticarcinogenic properties in humans.

Introduction

Xanthohumol (XN) is a prenylated flavonoid found in hop flowers and beer and alcohol-free refreshment drinks (1). Gerhäuser and coworkers studied its protective properties in in vitro assays and postulated that it is a promising cancer preventive agent due to its anti-initiating, anti-proliferative, and anti-oxidant properties (2). This assumption was supported by results of experiments with human hepatoma (HepG2) cells and with rat liver slices, which showed that low concentrations prevent induction of DNA damage by reactive oxygen species, by representatives of heterocyclic amines (HAA) also by polycyclic aromatic hydrocarbons (PAH; refs. 3–5). Also in experiments with mammalian cells and bacteria, antimutagenic effects were detected (6). Subsequent animal studies showed that XN is also active at low doses in rodents, i.e., inhibition of DNA damage and prevention of formation of neoplastic lesions which induced by the HAA 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) were observed in rats (7).

To find out if XN protects toward representatives of different groups of genotoxic carcinogens in humans, an experimental model was developed for the present study. Lymphocytes, which were collected from healthy participants of two intervention trials before and after consumption of the flavonoid, were exposed to representatives of different classes of dietary carcinogens which were activated with human-derived liver homogenate. After the treatment, DNA damage was monitored in single-cell gel electrophoresis (SCGE) assays which are based on the measurement of DNA migration in an electric field (8). This approach is increasingly used in human studies (9). The model carcinogens were nitrosodimethylamine (NDMA), [IQ], and benzo[a]pyrene (B[a]P). These agents are potential or proven human carcinogens, and their carcinogenic activities are caused by induction of damage of the genetic material (10–14). Additionally, we performed experiments in which the phosphorylation of histone 2AX was monitored, which reflects double-strand breaks. This method is increasingly used in human studies (15).

The first intervention study was a placebo-controlled trial with a cross-over design which was conducted with healthy individuals who consumed a XN-containing beverage for 2 weeks. To prove that the effects of the beverage are caused by the flavonoid, a follow-up study was realized in which the participants consumed XN in tablets.

Furthermore, attempts were made to elucidate the molecular mechanisms which account for the protective effects. Because earlier studies indicated that XN increases in rodents the activity...
of glutathione S-transferases (GST), which play a key role in the detoxification of different groups of xenobiotics (16), we monitored the impact of its consumption on the levels of 2 isoenzymes (α-GST and π-GST) and on the concentrations of glutathione (GSH).

Materials and Methods

Consumption of the XN drink

The beverage that was consumed by the participants is commercially not available and was provided by TA-XAN Company. One liter of the drink contained 12 mg of XN and 1.0 g roasted malt extract, 42 mg malic acid, and 40 g glucose. The placebo contained all components except XN. XN pills, which were used in the follow-up study, contained 12 mg of the flavonoid (XN, CAS Nr. 6754-58-1) and 422 mg lactose per pill. The chemicals were encapsulated with agarose.

Recruitment of the participants

The study was approved by the Ethical Commission of the Medical University of Vienna (EKNr.: 341/2010) and informed consent was obtained from all participants. Twenty-two individuals (men: 11; women: 11) participated in the main study; all of them consumed a mixed diet and were healthy non-smokers. None of them consumed pharmaceuticals (except contraceptives) or dietary supplements 3 weeks before and during the intervention and washout phase. Furthermore, the participants were asked not to consume beer and to reduce the intake of flavonoid rich foods and beverages (17) and to abstain from exhausting physical activities which may affect DNA stability (18, 19). The average body weight of the participants was 73.5 ± 12.8 kg (women: 66.2 ± 9.9; men: 80.8 ± 11.3), their BMI value 24.6 ± 3.3 kg/m² (women: 24.2 ± 3.6, men: 25.7 ± 3.1) and their age 26.1 ± 4.3 years (women: 27.3 ± 4.3, men: 25.0 ± 2.9).

Participants of the second trial (n = 10, women: 5 and men: 5) fulfilled the inclusion criteria; their body weights, BMI values, and age range were similar to those of the participants of the first trial.

Study design

The study had a cross-over design and was placebo controlled. After a run-in phase (4 days), half of the participants (n = 11) consumed either the XN-beverage or the placebo drink for 2 weeks (Fig. 1). Following a washout phase (2 weeks), the second part of the intervention was realized. During the intervention, each participant consumed 1 L of the beverage/d or an equal amount of the placebo.

Blood samples were collected before the consumption of the beverage, after 7 days and at the end of the 2 weeks’ intervention phase (4–5 hours after consumption of the beverage).

The second study, which was conducted with XN pills, had a parallel design. Ten participants (men: 5 and women: 5) consumed daily one pill (12 mg XN per pill) over a period of 2 weeks. As in the main study, they were asked to avoid consumption of beer, to control the intake of flavonoid rich foods, and to reduce their physical exercises.

The consumption of the beverage and of the pills did not cause any health problems in the participants according to information collected by questionnaires. All participants finished the study. Blood pressure and pulse frequencies were recorded at the beginning of the intervention and in weekly intervals. Both values were not altered after consumption of the XN beverage and by intake of the tablets (data not shown).

Sampling

Blood samples (60 mL/participant) were collected by venipuncture in heparinized tubes (Becton-Dickinson). After centrifugation (760 × g, 10 minutes, 4°C), the plasma samples were aliquoted and stored at −80°C. Lymphocytes were isolated with Histopaque 1077 (Sigma-Aldrich) by gradient centrifugation. Subsequently, suspensions of the cells were aliquoted in Biofreeze (Biochrom AG) and stored in liquid nitrogen.

Determination of DNA damage in lymphocytes with single-cell gel electrophoresis assays

The SCGE experiments were conducted under standard conditions according to the protocol of Hartmann and colleagues (20) and Burlinson and colleagues (21). Before the main study, experiments were conducted, in which induction of comet formation by the model carcinogens was monitored in lymphocytes from 3 healthy individuals (men: 2

![Figure 1](https://example.com/figure1.png)

Schematic representation of the study design.
and women: 1). Stock solutions of NDMA were prepared with PBS (pH 7.0), B(a)P, and IQ were dissolved in pure DMSO. NDMA and B(a)P were purchased from Sigma-Aldrich, IQ was from the Nard Institute (Tokyo, Japan). Each carcinogen was tested at different concentrations.

The lymphocytes were isolated by gradient centrifugation with Histopaque 1077 (Sigma-Aldrich). Suspensions of these cells were incubated with solutions of the test compounds and S9-mix, which was prepared from human liver homogenate (Trinova Biochem GmbH). The activation mix was composed according to the standard recipe of Maron and Ames (22) and had a protein content of 30.6 mg/mL. The incubation mixtures consisted of 890 μL PBS, 100 μL S9-mix, and 10 μL of solutions of the test compounds. Additionally, experiments were conducted with the mutagens, in which S9-mix was replaced by an identical amount of PBS.

After incubation of the mixtures at 37°C in the dark for 60 minutes, the suspensions were centrifuged (200 × g, 5 minutes, 4°C); subsequently, the cells were washed twice with PBS. Dead cells may cause false positive results in SCGE experiments (23); therefore, we determined the vitality of the cells with the trypan blue exclusion technique (23, 24).

Subsequently, comet assays were conducted under standard conditions (30-minute electrophoresis 300 mA, 1.0 V/cm, 4°C, pH ≥ 13) after lysis of the cells in alkaline buffer (pH 10.0) for 60 minutes. Then the slides were neutralized, air dried, and stained with ethidium bromide (2.0 μg/mL; Sigma-Aldrich; Supplementary Fig. S1).

From each sample, three slides were prepared and 50 cells were evaluated from each slide. Comet formation was analyzed with a fluorescence microscope (Nikon EFD-3) with 25-fold magnification. DNA migration was determined with a computer-aided image analysis system (Comet Assay IV, Perceptive Instruments). The percentage of DNA in tail was monitored as a parameter of comet formation. In the main experiments, comet measurements were conducted with the mutagens, in which S9-mix was replaced by an identical amount of PBS.

Statistical analysis

SCGE data from the main study with XN drink were evaluated by computing for each slide the median tail intensities of 50 cells; three slides per subject and condition were analyzed. Median tail intensities were arcsine transformed to remove correlations between means and standard deviations and to obtain normality of residuals. Data were analyzed by analysis of variance with placebo/XN-drink and time (baseline, days 7 and 14; Supplementary Table S2) as within-subject factors and sequence (placebo or XN-drink first) as a between subject factor. For the second intervention trial with pure XN, data were analyzed similarly but with only two time points (start, 14 days; Supplementary Table S2). Homogeneity of variances was tested by Levene’s tests and normality by Kolmogorov–Smirnov tests with Lilliefors’ corrected P values. Equality of covariance matrices was tested by Box-M tests. Comparison of mean values for days 7 and 14 against baseline levels was done by linear contrasts. Analysis was performed by Statistica 12.0 (StatSoft Inc.).

Results

Results that obtained with the model compounds in dose–response experiments with and without human liver S9-mix are shown in Fig. 2A–L. It can be seen that induction of DNA migration was found with all three carcinogens in SCGE experiments. With activation mix, the most pronounced effects were observed with B(a)P, which caused an increase of comet formation at a concentration of 2.5 μmol/L (Fig. 2E, P < 0.05). NDMA induced a clear effect under identical conditions when the cells were treated with levels > 20 μmol/L (Fig. 2A, P < 0.01). IQ was active when concentrations ≥ 1.0 mmol/L (Fig. 2I, P < 0.05) were added to the mixtures.

In experiments without S9 (Fig. 2C, G and K) positive results were obtained with NDMA (P < 0.01) and B(a)P (P < 0.05) with...
high dose levels, which exceeded the concentrations that we used in the main experiments. With IQ, only a marginal (not significant) effect was seen with levels ≤ 2 mmol/L.

In all experiments, the viability of the cells was determined after treatment of the cells, as cytotoxic effects may cause misleading results in SCGE experiments (23). It can be seen in Fig. 2B, F, J, D, H, and L that no alterations of the vitality were detected.

On the basis of the results of the dose response experiments, appropriate concentrations of the different carcinogens were selected for the main experiments.

The results that were obtained with cells which were collected at the start of the intervention and with samples which were collected after 7 and 14 days are depicted in Fig. 3A–F. Significant induction of DNA migration by the different carcinogens was observed in all experimental series (in all cases P < 0.001). The extent of DNA migration which was found in cells from participants who had consumed the placebo was not affected by the intervention (Fig. 3A, C and E).

A clear decrease of comet formation was observed after consumption of the XN drink in the experiments with B(a)P, with IQ (Fig. 3D and F, P < 0.001 after 7 and 14 days for both carcinogens). The extent of DNA migration was reduced already after intake of the XN drink for 1 week, prolonged consumption (14 days) led to an increase of the effects. In experiments with the nitrosamine a decline of comet formation was detected only at the end of the trial, but this effect was less pronounced (Fig. 3B, P = 0.196 after 7 days and P = 0.002 after 14 days).

We analyzed the DNA protective effects of XN also separately in males and females, but no gender-specific differences were detected. Furthermore, it is notable that the DNA protective effects which were seen at the end of the intervention phase I disappeared completely after the washout interval (data not shown). However, after consumption of the placebo drink, a significant increase of tail intensities was detected (Fig. 3B, P = 0.006 after 7 days and P = 0.002 after 14 days).

Figure 2.
A–L, Induction of DNA damage by NDMA, B(a)P, and IQ in human lymphocytes. The cells were collected from 3 donors (men: 2 and woman: 1) and treated separately with different doses of the test compounds in the presence of human derived liver SJ-mix for 60 minutes. Subsequently, DNA migration was monitored under standard conditions (A, E and I). The compounds were also tested in the absence of activation mix (C, G, and K). The viability of the cells was determined with trypan blue (B, F, J, D, H, and L). Per experimental point, 3 cultures were made in parallel. From each incubation mix, 3 slides were made and 50 cells were evaluated per slide. Bars represent means and SDs. C indicates solvent controls [PBS for NDMA, DMSO for B(a)P, and IQ]. P ≤ 0.05 were considered as significant in comparison with control values and are marked with asterisks (ANOVA followed by the Dunnett multiple comparison test: *, P < 0.05; **, P < 0.01; ***, P < 0.001).
Figure 3.
A–F, impact of consumption of the XN beverage on DNA damage and on the vitality of the cells. Lymphocytes that had been collected before, during, and after consumption of the XN beverage or of a placebo were exposed to NDMA (30.0 μmol/L; A and B), B(a)P (5.0 μmol/L; C and D) or IQ (1.0 mmol/L; E and F) in presence of human liver S9-mix for 60 minutes. Subsequently, DNA migration was measured under standard conditions, and the vitality of the cells was determined with trypan blue. Data plot %DNA in tail for each of 22 individuals included in the cross-over experiment with placebo and XN-drink conditions before and after 7 and 14 days with a washout period of 14 days in between. Data points represent averages of 3 slides from which the median tail intensities were computed and arcsine transformed. Horizontal lines represent means of results obtained with 22 subjects, boxes' ranges of 2 standard errors. Shown are control conditions and treatment of cells with NDMA (A, B), B(a)P (C, D) or IQ (E, F). Significant differences to baseline are indicated by stars (*, P < 0.05; **, P < 0.01; ***, P < 0.001), they are obtained by linear contrasts after repeated measurements with ANOVA.
differences to baseline, indicated by asterisks (\( \ast \)). DNA migration was monitored after treatment of the cells with B(a)P or IQ. Lymphocytes were collected at the start and at the end of the intervention. Women: 5) consumed XN tablets (12 mg/tablet, 1 tablet/P/d) over 14 days. After 14 days, consumption of 12 mg XN, the chemically induced effects declined (B(a)P: 15.43, IQ: 11.32; control: 6.31). All values are means \pm SDs of results obtained with 10 participants. The effects that we found after consumption of XN were not unexpected. As mentioned above, evidence for protective effects toward HAAs and PHAs was seen in earlier in vitro studies with human and rodent liver cell lines (3–5). These findings were not unexpected as we found in earlier studies that the compounds that we used induce comet formation without liver-derived metabolic activation mix in peripheral lymphocytes at high concentrations; the mechanisms that cause these effects are described by Nersesyan and colleagues (9).

All carcinogens that we tested are contained in foods and cause cancer in laboratory animals, and it is likely that they are involved in the etiology of human cancer (10–12). In the case of B(a)P and structurally related compounds, inhalative exposure at workplaces and via tobacco consumption and environmental contaminations as well as consumption of contaminated foods may lead to adverse effects in humans (12). All compounds induce DNA damage in humans and in rodents, and it was postulated that the induction of cancer by these chemicals is a consequence of their genotoxic properties (10–12). NDMA is converted to highly DNA-reactive methyl-diazonium ions (28), from B(a)P and other PAHs reactive diol epoxides are formed, which attack DNA bases (12); the ultimate carcinogens of IQ and other HAAs are genotoxic arylnitrenium derivatives (29).

Figure 4. A–C, impact of consumption of XN pills on induction of DNA migration in human lymphocytes which were treated with B(a)P or IQ (A). The participants (men: 5; women: 5) consumed XN tablets (12 mg/tablet; 1 tablet/P/d) over 14 days. Lymphocytes were collected at the start and at the end of the intervention. DNA migration was monitored after treatment of the cells with B(a)P or IQ under identical conditions, as in the experiment with the XN drink (see legend of Fig. 3). Each donor, 3 cultures were set up in parallel. Per culture one slide was prepared and 50 cells were evaluated per slide. Significant differences to baseline, indicated by asterisks (\( \ast \), \( \ast \ast \), \( \ast \ast \ast \)), were obtained by linear contrasts after repeated measurements with ANOVA.

Additionally, \( \gamma \)H2AX experiments were conducted to confirm effects of pure XN toward B(a)P and IQ with another method. Indeed, we also found with this approach reduction of DNA damage after XN consumption. B(a)P and IQ caused a significant increase of histone phosphorylation before XN consumption (B(a)P: 15.43 \pm 4.78; IQ: 11.32 \pm 6.17, control value: 6.31 \pm 5.23). After 14 days, consumption of 12 mg XN, the chemically induced effects declined (B(a)P: 8.14 \pm 2.53; IQ: 7.13 \pm 3.98, control value: 4.11 \pm 2.68). All values are means \pm SDs of results obtained with 10 participants.

Figures 5A and B depict the results of experiments that were conducted to elucidate the mechanisms that cause the protective effects. The measurements were conducted with plasma samples which were collected in the main study. It can be seen that a significant (ca. 42.8 %, \( P < 0.05 \)) increase of \( \pi \)-GST concentrations was observed in the XN group but not in the placebo group while the levels of \( \pi \)-GST were not altered; furthermore, no changes of the overall GST activity and of the GSH concentrations were found (data not shown).

Discussion

This article describes the development of an \textit{ex vivo} model that allows to study the impact of dietary interventions on chemically induced DNA instability in humans. We used it to investigate the chemopreventive activities of the hop flavonoid XN toward representatives of three major groups of dietary carcinogens (nitrosamines, PAHs, and HAAs) and found protective effects toward B(a)P and IQ and also a moderate reduction of NDMA-induced DNA damage.

The concentrations of the model compounds that were used in the main trial with human-derived liver S9 were low and were determined in dose range experiments (Fig. 2A, E and I). In the absence of activation mix, induction of DNA damage was seen under identical conditions only with (unphysiological) high concentrations (Fig. 2C, G and K). These findings were not unexpected as we found in earlier studies that the compounds that we used induce comet formation without liver-derived metabolic activation mix in peripheral lymphocytes at high concentrations; the mechanisms that cause these effects are described by Nersesyan and colleagues (9).

The effects that we found after consumption of XN were not unexpected. As mentioned above, evidence for protective effects toward HAAs and PHAs was seen in earlier in vitro studies with human and rodent liver cell lines (3–5). These latter results confirm the effects that were seen in the "comet" experiments. Furthermore, it is notable that reduction of IQ-induced DNA migration and formation of preneoplastic lesions were found in earlier experiments with rats after treatment with low doses of flavonoid (7). It is notable that the overall baseline values of the placebo group are lower than those of the XN group (Fig. 3; Supplementary Table S1). A possible explanation for this phenomenon is seasonal effects that were also observed in earlier intervention trials that last over several weeks (9). A closer look at the results of the present study show that this effect was partly due to the fact that increased DNA migration was seen in the individuals who consumed first the placebo drink at the end of the washout period, while no increased values were found in participants who consumed XN first. This difference may be indicative for a protective effect of XN toward the increase caused by seasonal effects. Hence, protection from DNA damage seems to extend over a longer period than 14 days, which may reflect the longer life cycles of subpopulations of peripheral lymphocytes. It is important to note that the difference between the placebo and the XN group has no impact on the protective effects observed with the different model carcinogens.

The results of the measurements of the biochemical parameters show that the flavonoid causes significant induction of \( \alpha \)-GST while the \( \pi \)-GST was not altered. This observation provides an explanation for the protective effects towards B(a)P as well as apurinic sites (8); additionally, we also performed H2AX experiments, which reflect double-strand breaks. These latter results confirm the effects that were seen in the "comet" experiments. Furthermore, it is notable that reduction of IQ-induced DNA migration and formation of preneoplastic lesions were found in earlier experiments with rats after treatment with low doses of flavonoid (7). It is notable that the overall baseline values of the placebo group are lower than those of the XN group (Fig. 3; Supplementary Table S1). A possible explanation for this phenomenon is seasonal effects that were also observed in earlier intervention trials that last over several weeks (9). A closer look at the results of the present study show that this effect was partly due to the fact that increased DNA migration was seen in the individuals who consumed first the placebo drink at the end of the washout period, while no increased values were found in participants who consumed XN first. This difference may be indicative for a protective effect of XN toward the increase caused by seasonal effects. Hence, protection from DNA damage seems to extend over a longer period than 14 days, which may reflect the longer life cycles of subpopulations of peripheral lymphocytes. It is important to note that the difference between the placebo and the XN group has no impact on the protective effects observed with the different model carcinogens.

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it is known that the diol-epoxide of B(a)P is a substrate for human class α-GSTs (31). It is also documented that α-GST catalyzes the detoxification of diol-epoxides of a variety of other PAHs and also of other DNA-reactive chemicals such as peroxides, aflatoxins, and styrene oxide (31–33). In regard to the effects that were observed with IQ, it is notable that it is not known if the metabolites of HAAs are substrates for α-GST in general, but there is evidence that they are involved in the detoxification of PhIP, which is a structurally related “cooked food mutagen” (31, 34).

A number of investigations showed that human α-GST is induced by dietary components that protect against cancer such as Brassica vegetables, tea polyphenols, and coffee (32, 35). In this context, it is also notable that polymorphisms of genes encoding for human class α-GSTs, which reduce the activity of the enzyme, are associated with increased risks for specific forms of cancer, in particular in the colon (35–38). These observations support the assumption that increased α-GST levels lead to protection against different forms of cancer.

The molecular mechanisms that cause upregulation of the transcription of genes encoding for α-GST are only partly known (32). Nrfl2 plays a key role in the synthesis of phase II enzymes, including GSTs; and it is notable that Lee and coworkers (39) demonstrated that XN causes induction of antioxidant enzymes via activation of this transcription factor in neuronal cells.

Apart from the interaction of XN with the activities of drug-metabolizing enzymes, it is also possible that protection against genetic damage is caused by activation of DNA repair. Such effects have been found with a number of dietary compounds (40). However, no repair effects were investigated in experiments with XN. It is notable that inhibition of DNA topoisomerase I was detected in XN experiments with different cell lines (41). It is known that this topoisomerase inhibition affects DNA repair but extremely high XN doses were used in these in vitro experiments that caused acute cytotoxic effects. Therefore, this observation is probably not relevant for the present study.

It is notable that the experimental model that we developed for this study can provide valuable information on chemopreventive properties of other dietary constituents and pharmaceuticals toward a variety of human carcinogens.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: F. Ferk, S. Knasmueller
Development of methodology: C. Pichler, M. Kundl
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Pichler, W. Huber, W. Jäger, M. Waldherr, A. Nesseyan, I. Herbaček
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Pichler, H. Al-Serori, W. Huber, W. Jäger, M. Misić, M. Kundl, I. Herbacek
Writing, review, and/or revision of the manuscript: W. Huber, S. Knasmueller
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Al-Serori, A. Nesseyan
Study supervision: S. Knasmueller
Other (Comet assay evaluation; α-GST and π-GST measurements): C. Pichler

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**Figure 5.**  
A and B, Impact of consumption of the XN-supplemented drink on the activities of α-GST (A) and π-GST (B) in plasma. Bars represent means and standard deviations of samples which were collected in the first intervention trial (n = 22). Asterisks indicate statistical significance (Students t test, * P < 0.05).
References

Cancer Prevention Research

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