Published in final edited form as:

Chem Biol Interact. 2013 October 25; 206(1): . doi:10.1016/j.cbi.2013.07.010.

Evidence for Endogenous Formation of the Hepatocarcinogen *N*-Nitrosodihydrouracil in Rats Treated with Dihydrouracil and Sodium Nitrite: a Potential Source of Human Hepatic DNA Carboxyethylation

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Abstract

An earlier study demonstrated that hydrolysates of all human liver DNA samples analyzed contain the DNA adduct 7-(2 -carboxyethyl)guanine (7-CEGua) with an average level of 74.6 adducts per 10⁹ nucleotides. One possible source of this DNA adduct would be endogenous nitrosation of the normal pyrimidine metabolites dihydrouracil (DHU) and -ureidopropionic acid (-UPA), yielding the corresponding nitroso compounds *N*-nitrosodihydrouracil, a potent hepatocarcinogen, and *N*-nitroso- -ureidopropionic acid. Another potential source would be reaction of endogenously formed acrylic acid with DNA. We tested these hypotheses in a study in which rats were treated with NaNO₂ in the drinking water, alone, or in combination with dietary DHU or -UPA, or with acrylic acid in the drinking water, for either 2 or 4 weeks. Hepatic DNA from these rats was analyzed for 7-CEGua, using liquid chromatography-tandem mass spectrometry-selected reaction monitoring with confirmation by high resolution mass spectrometry. The results demonstrated consistent statistically significant increases of 7-CEGua in hepatic DNA of the rats treated with the combination of NaNO₂ and DHU compared to the corresponding controls, while the other treatments gave variable results. These results support the hypothesis that endogenous nitrosation of DHU could be a major source of 7-CEGua in human hepatic DNA. Development of methodology for analysis of 7-CEGua in human leukocyte DNA is also reported, which will allow testing of this hypothesis in epidemiologic and clinical studies.

Keywords

7-carboxyethylguanine; dihydrouracil; -ureidopropionic acid; acrylic acid; endogenous nitrosation; *N*-nitrosodihydrouracil

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Conflict of interest

The authors declare that there is no conflict of interest

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1. Introduction

Liver cancer is expected to cause approximately 20,000 deaths in the U.S. in 2012 [1]. It is often accompanied by cirrhosis. Known etiologic factors for liver cancer worldwide, where it is responsible for over 500,000 deaths per year and is the 3rd most frequent cause of cancer death, include hepatitis B and C virus infection, exposure to aflatoxins, alcohol consumption, and tobacco smoking [2]. Liver cancer and cirrhosis have these causative factors in common. Among known causes, only hepatitis C virus, alcohol, and tobacco smoking are likely to be important etiologic factors in the U.S. and other Western countries. This raises a crucial question with respect to this rapidly fatal disease: is there a common cause of liver cancer that has been previously overlooked?

It is axiomatic that DNA adducts are involved in carcinogenesis [3;4]. Convincing data demonstrate that DNA adducts, if unrepaired by cellular DNA repair enzymes, can cause miscoding during replication [4;5]. If the resulting mutations occur in critical regions of important growth control genes such as *KRAS* or *TP53*, the result can be loss of normal cellular growth control mechanisms, genomic instability, and cancer [6]. Powerful genotoxic carcinogens such as *N*-nitrosoureas, *N*-nitrosamines, polycyclic aromatic hydrocarbons, and aflatoxins certainly initiate carcinogenesis by this general mechanism [7–10]. One approach to investigating etiologic factors in cancer is to work backwards from DNA adduct structures to the potentially responsible carcinogen. That general strategy forms the basis for the study described here.

We have previously reported that the DNA adduct 7-(2 -carboxyethyl)guanine (7-CEGua, **7**, Scheme 1) was present in hydrolysates of all 24 human liver DNA samples analyzed, with levels ranging from 17 – 1189 fmol/μmol Gua, and a mean ± SD of 373 ± 320 fmol/μmol Gua (74.6 adducts per 10⁹ nucleotides) [11]. One known source of 7-CEGua is *N*-nitrosodihydrouracil (NDHU, **4**). Following treatment of rats with NDHU, 7-CEGua was detected in hydrolysates of hepatic DNA [12]. This resulted from hydrolysis of NDHU in vivo, leading via *N*-nitroso- -ureidopropionic acid (*N*- -UPA, **5**) to the alkylating intermediate, 2-carboxyethyldiazonium ion (**6**) and consequent carboxyethylation of deoxyguanosine at its reactive 7-position, yielding 7-CEGua (**7**) after hydrolysis of DNA. NDHU is a powerful hepatocarcinogen when administered orally to rats; it also induced some kidney tumors [13;14]. Thus, Bulay et al demonstrated that treatment of rats with 45 ppm NDHU in the drinking water resulted in a 96% incidence of hepatocellular carcinoma with a latency period of 45 ± 8 weeks [14]. Collectively, these results demonstrate that one source of 7-CEGua in hydrolysates of hepatic DNA could be the hepatocarcinogen NDHU.

A plausible source of human exposure to NDHU is endogenous nitrosation of dihydrouracil (DHU, **2**, Scheme 1). Endogenous nitrosation can occur in humans when salivary nitrite reacts with amines or amides ingested in the diet or present in the body [15]. This has been clearly demonstrated in studies which showed significant increases in *N*-nitrosoproline in human urine following exposure to nitrite and proline [15]. DHU is a normal intermediate in mammalian metabolism, formed from uracil (**1**) by dihydropyrimidine dehydrogenase [16;17]. DHU is present in human plasma [17;18] and urine [16–19]. Levels of DHU excreted in the urine of healthy humans have been reported to range from about 2 – 10 mg/day. Furthermore, DHU is metabolized to -ureidopropionic acid (-UPA, **3**), which is found at similar levels as DHU in urine [16;19], and could also be nitrosated, giving rise to *N*- -UPA (**5**). Thus, both DHU and -UPA could be converted to the 2-carboxyethyldiazonium ion upon nitrosation (Scheme 1).

In this study, we tested the hypothesis that 7-CEGua levels in hydrolysates of rat liver DNA could increase upon treatment of rats with NaNO₂ plus DHU or -UPA. If this hypothesis

were correct, then endogenous nitrosation of DHU to NDHU, or -UPA to N-UPA, might be a source of 7-CEGua in human liver DNA, and this process could potentially be involved in liver cancer etiology. We also examined the possibility that 7-CEGua adducts in hydrolysates of hepatic DNA might result from treatment of rats with acrylic acid, which reacts slowly with DNA in vitro, resulting in formation of 7-CEGua [20].

2. Materials and Methods

2.1 Chemicals

DHU, NaNO₂, and acrylic acid were purchased from Sigma-Aldrich (St. Louis, MO). -UPA was obtained from Chem-Impex International Inc (Wooddale, IL).

2.2 Animal study

This study was approved by the University of Minnesota Institutional Animal Care and Use Committee. Male F-344 rats, age 6 weeks, were obtained from Charles River Laboratories (Kingston, NY), housed 2 per cage with Harlan irradiated corn cob bedding (Harlan, Indianapolis IN), and allowed to acclimate to the Research Animal Resources facility, University of Minnesota, for one week. The rats were maintained under standard conditions (20–24 °C temperature, 29–32% relative humidity and 14/10 light/dark cycle). They were fed Harlan Teklad 7022 (NIH-07) diet. Water and diet consumption were measured three times per week.

In Study 1, 4 rats per group, except as noted, were treated for 2 weeks as follows: 1, NaNO₂ 1500 ppm in drinking water; 2, DHU 2480 ppm in powdered diet; 3, NaNO₂ 1500 ppm + DHU 2480 ppm; 4, -UPA 2870 ppm in powdered diet; 5, NaNO₂ 1500 ppm + -UPA 2870 ppm; 6, acrylic acid 1565 ppm in drinking water, 6 rats; 7, untreated control, 6 rats. The rats were humanely sacrificed by CO₂ overdose, and tissues were collected and frozen at –20 °C until analysis.

In Study 2, 4 rats per treatment group were given the compounds for 4 weeks exactly as in Study 1, except that one additional group was added with a higher dose of acrylic acid (3130 ppm). There were 6 rats in the control group. The rats were sacrificed and tissues collected and frozen as in Study 1.

2.3 Analysis of rat hepatic DNA hydrolysates for 7-CEGua by liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC-ESI-MS/MS-SRM)

DNA isolation and enzymatic hydrolysis were carried out essentially as described [11]. In brief, for hydrolysis, DNA (1 mg) was dissolved in 1 mL of 10 mM Tris-HCl/5 mM MgCl₂ buffer (pH 7.0) containing [¹⁵N₅]7-CEGua (1300 fmol). The DNA was enzymatically hydrolyzed for 70 min at 37 °C with 1060 units of DNase I (type II, bovine pancreas), 0.05 units of phosphodiesterase I (type II, *Crotalus adamanteus* venom), and 300 units of alkaline phosphatase (calf intestine).

The hydrolysate, after removal of a 10 µL aliquot for dGuo quantitation, was purified using a mixed mode cation exchange cartridge [MCX Vac RC, 60 mg (Waters Corp, Milford, MA)]. The cartridge was conditioned with 2 mL of CH₃OH and 2 mL 2% H₃PO₄. The sample was acidified with 10 µL of 86% H₃PO₄. After the sample was applied, the cartridge was washed with 2 mL 0.1% H₃PO₄ and 2 mL of CH₃OH, and the analyte was eluted with 2 mL 3% NH₄OH in CH₃OH. This fraction was collected and concentrated to dryness. One mL of a freshly prepared 10% CH₃COCl solution in CH₃OH was added to the vial. The mixture was then heated for 1 h at 50 °C to convert 7-CEGua to its methyl ester, then

concentrated to dryness. The residue was dissolved in 1 mL 15 mM NH₄OAc buffer (pH 6.6) and purified using a Strata-X solid-phase extraction cartridge [33 μ m, 30 mg/1 mL (Phenomenex, Torrance, CA)]. The cartridge was conditioned with 1 mL CH₃OH, 1 mL H₂O and 1 mL 15 mM NH₄OAc buffer (pH 6.6). After the sample was applied, the cartridge was washed with 1 mL 15 mM NH₄OAc buffer (pH 6.6), 1 mL H₂O, and 1 mL 2% CH₃OH. Finally the analyte was eluted with 1 mL of 80% CH₃OH, this fraction was collected and evaporated to dryness. The residue was dissolved in 40 μ L of 15 mM NH₄OAc buffer (pH 6.6), and 8 μ L aliquots were injected and analyzed by LC-ESI-MS/MS-SRM.

Adduct analysis by LC-ESI-MS/MS-SRM was carried out with a TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA) interfaced with an Agilent 1100 capillary flow HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 0.5 x 150 mm Hypersil Gold PFP column (Thermo). The column was operated at 30 °C and a flow rate of 10 μ L/min. A 10 min linear gradient from 2% to 35% CH₃CN in 15 mM NH₄OAc buffer (pH 6.6) was followed by a 35% CH₃CN hold for 5 min, and then by a 2 min gradient from 35 to 80% CH₃CN. The column was washed for 3 min with 80% CH₃CN, then returned to 2% CH₃CN in 2 min and finally re-equilibrated for 15 min. The MS parameters were set as follows: spray voltage, 4 kV; sheath gas pressure, 30; capillary temperature, 250 °C; collision energy, 22 V; scan width, 0.1 amu; scan time, 0.4 s; Q1 peak width, 0.7; Q3 peak width, 0.7; Q2 pressure, 1.0 mTorr; source CID, 8V; and tube lens offset, 95V. Transitions monitored were as follows: m/z 238 [M + H]⁺ m/z 152 [BH]⁺ for 7-CEGua methyl ester; and m/z 243 [M + H]⁺ m/z 157 [BH]⁺ for [¹⁵N₅]7-CEGua methyl ester.

Calibration curves were constructed before each analysis using standard solutions of 7-CEGua and [¹⁵N₅]7-CEGua. A constant amount of [¹⁵N₅]7-CEGua (1300 fmol) was mixed with differing amounts of 7-CEGua (10, 20, 40, 60, 100, and 200 fmol), and these were esterified with CH₃COCl and CH₃OH and analyzed by LC-ESI-MS/MS-SRM. Each set of rat hepatic DNA samples contained negative (buffer blanks) and positive (calf thymus DNA samples) controls.

2.4 Isolation of human leukocyte DNA

This study was approved by the University of Minnesota Institutional Review Board. Blood samples were obtained by venipuncture from 5 non-smokers. Leukocytes were isolated and DNA was extracted as previously reported [21]. Briefly, DNA was isolated using the DNA purification from buffy coat protocol (Qiagen Corp. Valencia CA) with several modifications. Three mL of RBC cell lysis solution was added to 1 mL of buffy coat prepared from 10 mL of whole blood. The white blood cell pellet was collected by centrifugation and treated with 5 mL of cell lysis solution and 50 μ L of RNase A (4 mg/mL). To the cell lysate was added 2 mL of protein precipitation solution, and the mixture was centrifuged to remove protein. DNA was precipitated from the supernatant by the addition of 5 mL of isopropanol. The DNA was then washed with 2 mL of 70% ethanol in H₂O and then 100% ethanol. DNA was dried in a stream of N₂ and stored at -20°C until use. DNA hydrolysis was carried out as described in Section 2.3.

2.5 Analysis of DNA hydrolysates for 7-CEGua by liquid chromatography-nanoelectrospray ionization-high resolution tandem mass spectrometry (LC-NSI-HRMS/MS)

Rat and human samples which had been purified and derivatized as described in Section 2.3 were re-suspended in 10 μ L of H₂O. The amounts corresponded to an average DNA concentration of about 26 μ g/ μ L. Separation was performed on a Nano2D-LC HPLC (Eksigent, Dublin, CA) system equipped with a 1 μ L injection loop. One μ L of sample was

injected onto a capillary column (75 μ m ID, 10 cm length, 15 μ m orifice) created by hand packing a commercially available fused-silica emitter (New Objective, Woburn MA) with Luna C18 bonded separation media (Phenomenex, Torrance, CA). The flow rate was 300 nL/min with a 15 min hold at 98% 15 mM ammonium acetate buffer followed by a 10 min linear gradient from 2 to 50% CH₃CN, followed by a 5.5 min re-equilibration at 1000 nL/min of 2% CH₃CN. Samples were analyzed by nanoelectrospray using an LTQ-Orbitrap Velos instrument (Thermo Scientific, Waltham, MA). The nanoelectrospray source voltage was set at 1.6 kV. The capillary temperature was 350 °C and the S-lens RF level was set at 40%. Adducts were quantified by HRMS/MS of 7-CEGua methyl ester at m/z 238 m/z 152.0567 and of [¹⁵N₅]7-CEGua methyl ester at m/z 243 m/z 157.0419 with accurate mass monitoring of the fragment ions at 5 ppm mass tolerance (152.0567 $\pm$ 0.0008 and 157.0419 $\pm$ 0.0008 respectively) utilizing the Orbitrap detector. These two MS/MS events were performed using the HCD collision cell with a 0.54 amu isolation width, collision energy of 50% and the resolution set at 30,000 (at 400 amu) with an actual resolution of 55,000 (at 152 and 157 amu).

A calibration curve was constructed before each analysis using a standard solution of 7-CEGua and [¹⁵N₅]7-CEGua. A constant amount of [¹⁵N₅]7-CEGua (10 fmol) was mixed with various amounts of 7-CEGua (0.1, 0.5, 1, 2, and 4 fmol), derivatized to their methyl esters, and analyzed.

2.6 HPLC-UV analysis for quantitation of dGuo and Gua

This was performed with an Agilent 1100 capillary flow HPLC with a diode array detector set at 254 nm (Agilent Technologies, Palo Alto, CA). A 0.5 $\times$ 250 mm Luna 5 μ m C18 column (Phenomenex, Torrance, CA) was used with a gradient from 5 to 22% CH₃OH in H₂O over the course of 20 min at a flow rate of 10 μ L/min. This system was used for quantitation of dGuo in hydrolysates of DNA samples. Gua values reported in the results were calculated from the measured dGuo.

2.7 Statistical analysis

Statistical analysis of 7-CEGua levels was performed using a one-way analysis of variance (ANOVA) approach, and pairwise comparisons were conducted controlling for the false discovery rate at a 5% level [22].

3. Results

Body weights, diet and water consumption, and daily doses per rat of the test compounds in Studies 1 and 2 are summarized in Tables 1 and 2, respectively. In a 14-week study in male rats carried out by the U.S. National Toxicology Program, the dose of NaNO₂ used here, 1500 ppm in the drinking water, did not affect body weights and showed little toxicity. The same dose of NaNO₂ was not carcinogenic in a 2-year study [23]. We chose this dose to maximize the chances of detecting endogenous nitrosation if it did occur. The doses of DHU, -UPA, and the lower dose of acrylic acid were chosen to approximate the total NaNO₂ dose on a molar basis. An additional group in the 4 week study received a higher dose of acrylic acid (Table 2).

Hepatic DNA was hydrolyzed and analyzed for 7-CEGua as its methyl ester, using the method which we have described previously with slight modifications [11]. LC-ESI-MS/MS-SRM chromatograms from this analysis are illustrated in Figure 1 for hydrolysates of hepatic DNA from control rats, rats treated with DHU only, or rats treated with DHU plus NaNO₂.

We sought further evidence for the identity of 7-CEGua in some samples of hepatic DNA by LC-NSI-HRMS/MS, using the transitions m/z 238 $\rightarrow$ m/z 152.0567 for 7-CEGua methyl ester and at m/z 243 $\rightarrow$ m/z 157.0419 for [$^{15}\text{N}_5$]7-CEGua methyl ester. A typical chromatogram from this analysis is illustrated in Figure 2. These results confirmed the identity of 7-CEGua in rat hepatic DNA in this experiment and demonstrate the increased selectivity of the LC-NSI-HRMS/MS method.

The results of the analyses of rat hepatic DNA from Studies 1 and 2 are summarized in Table 3. In both studies, statistically significant increases in levels of 7-CEGua were observed in hepatic DNA of rats treated with NaNO_2 plus DHU compared to the rats treated with NaNO_2 or DHU alone. In Study 1, in which treatment was for 2 weeks, these increases in 7-CEGua were 1.7 – 1.9 fold while in Study 2, with 4 weeks of treatment, they were 3.8 – 3.9 fold. None of the other treatments gave statistically significant increases in levels of 7-CEGua compared to the appropriate controls in both the 2 and 4 week studies. In the rats treated with -UPA and NaNO_2 , significant increases in 7-CEGua were seen only in the 4 week study. Similarly, acrylic acid treatment produced significant increases only in the 4 week study.

We extended our LC-NSI-HRMS/MS methodology to the analysis of human leukocyte DNA since this would ultimately be necessary for investigating levels of 7-CEGua in humans. We were able to obtain clear evidence for the presence of 7-CEGua in all 5 human leukocyte DNA samples examined, as shown in Figure 3. The average amount of 7-CEGua was 103 ± 89 fmol/ μmol Gua.

4. Discussion

The results of this study clearly demonstrate the potential of endogenous nitrosation of DHU as a source of 7-CEGua in hydrolysates of hepatic DNA. Treatment of rats with DHU in the diet and NaNO_2 in the drinking water resulted in significant increases in 7-CEGua in hydrolysates of liver DNA compared to control rats treated only with DHU or NaNO_2 . Levels of 7-CEGua in hydrolysates of hepatic DNA were determined by LC-MS/MS-SRM and confirmed by LC-NSI-HR-MS/MS analysis. The formation of NDHU most likely occurred in the stomach. These results provide a solid foundation for further exploration of the hypothesis that 7-CEGua, present in all human hepatic DNA hydrolysate samples examined, could originate from DNA carboxyethylation by the powerful hepatocarcinogen NDHU, which in turn could result from endogenous nitrosation of the pyrimidine metabolite DHU.

DHU has been detected in human plasma and urine [16–19]. There is great inter-individual variation in endogenous DHU/uracil ratios, with some individuals having relatively high levels of DHU [24]. It is plausible that such individuals may be at high risk for endogenous formation of NDHU.

The most favorable conditions for endogenous formation of NDHU would occur in the stomach, where acidic pH favors the nitrosation reaction, and nitrosation of DHU to NDHU proceeds at a moderate rate compared to other amides [25]. There is no reason to believe that metabolically formed endogenous DHU would concentrate in the stomach. Therefore, an important question concerns exogenous sources of DHU, specifically its possible presence in the human diet. Since nitrite is commonly found in the diet and is present in human saliva, formation of NDHU from dietary DHU is clearly feasible, as we demonstrated. We are not aware of any studies which have assessed dietary exposure to DHU, but its occurrence in meat products would be plausible.

We used LC-NSI-HRMS/MS to confirm the identity of 7-CEGua in hydrolysates of rat hepatic DNA in this study. This technique is even more discriminating than LC-ESI-MS/MS-SRM because the Orbitrap technology allows measurement of m/z with great accuracy, providing high resolution MS data that eliminate ambiguity in assignment of the elemental composition of a selected ion, in this case, m/z 152.0567, representing loss of -CH₂CH₂COOCH₃ from 7-CEGua, with proton transfer to Gua. The result is a clearer chromatogram, as can be seen by comparing Figures 1 and 2, because virtually all background is eliminated. We were not able to use LC-NSI-HR-MS/MS for all of the analyses in this study because of instrument availability. The LC-NSI-HR-MS/MS technique has great potential for future studies on the formation of 7-CEGua in humans because, as shown in Figure 3, its increased selectivity and sensitivity allows clear detection of this analyte in human leukocyte DNA, readily available from clinical and epidemiologic studies. This is the first example of 7-CEGua in hydrolysates of human leukocyte DNA, as our previous study quantified this adduct only in hydrolysates of human hepatic DNA [11]. The ability to quantify 7-CEGua in human leukocyte DNA should allow examination of the endogenous nitrosation hypothesis in future studies comparing hepatic and leukocyte DNA adduct levels in rats and examining leukocyte DNA adduct levels in humans.

The mutagenic consequences of 7-CEGua are unknown, but 7-substituted deoxyguanosines in DNA depurinate readily, leading to abasic sites. Abasic sites have been implicated as causes of G → T mutations and other changes [26;27].

In summary, the results of this study support the hypothesis that the hepatocarcinogen NDHU can form endogenously from the pyrimidine metabolite DHU and nitrite, resulting in significant increases in levels of 7-CEGua in hydrolysates of hepatic DNA. Further studies are required to determine if endogenous nitrosation of DHU is the source of 7-CEGua observed in DNA of all human liver samples analyzed to date.

Acknowledgments

Funding Source

This study was supported by grants CA-85702 and CA-81301 from the U.S. National Cancer Institute. Mass spectrometry was carried out in the Analytical Biochemistry Shared Resource of the Masonic Cancer Center, University of Minnesota, supported in part by Cancer Center Support Grant CA-77598.

We thank Bob Carlson for editorial assistance.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin.* 2012; 62:10–29. [PubMed: 22237781]
2. Boyle, P.; Levin, P. World Cancer Report, 2008. IARC; Lyon, FR: 2008. p. 350-356.
3. Miller JA, Miller EC. Chemical carcinogenesis: mechanisms and approaches to its control. *J Natl Cancer Inst.* 1971; 47:5–14.
4. Shrivastav N, Li D, Essigmann JM. Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation. *Carcinogenesis.* 2010; 31:59–70. [PubMed: 19875697]
5. Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA. Environmental and chemical carcinogenesis. *Semin Cancer Biol.* 2004; 14:473–486. [PubMed: 15489140]
6. Weinberg, RA. *The Biology of Cancer.* Garland Science; New York: 2007.
7. Preussmann, R.; Stewart, BW. *N-Nitroso Carcinogens.* In: Searle, CE., editor. *Chemical Carcinogens.* 2. American Chemical Society; Washington, DC: 1984. p. 643-828. ACS Monograph 182

8. Dipple, A.; Moschel, RC.; Bigger, CAH. Polynuclear aromatic hydrocarbons. In: Searle, CE., editor. Chemical Carcinogens. 2. American Chemical Society; Washington, D.C: 1984. p. 41-163.ACS Monograph 182
9. Kensler, TW.; Groopman, JD. Carcinogenic mycotoxins. In: Bowden, GT.; Fischer, SM., editors. Comprehensive Toxicology: Chemical Carcinogens and Anticarcinogens. 1997. p. 201-223.
10. Hecht, SS. *N*-Nitrosamines. In: Rom, WB.; Markowitz, SB., editors. Environmental and Occupational Medicine. 4. Wolters Kluwer/Lippincott Williams & Wilkins; Philadelphia: 2007. p. 1226-1239.
11. Cheng G, Wang M, Villalta PW, Hecht SS. Detection of 7-(2 -carboxyethyl)guanine but not 7-carboxymethylguanine in human liver DNA. *Chem Res Toxicol*. 2010; 23:1089–1096. [PubMed: 20438065]
12. Mirvish SS, Ross AE, Gold B, Drake N. In vitro and in vivo formation of 7-(2 -carboxyethyl)guanine from the liver carcinogen 1-nitroso-5,6-dihydrouracil and its reactions with water and methanol. *J Natl Cancer Inst*. 1985; 74:1105–1110. [PubMed: 2582174]
13. Mirvish SS, Garcia H. 1-Nitroso-5,6-Dihydrouracil: Induction of Liver Cell Carcinomas and Kidney Adenomas in the Rat. *Krebsforsch Klin Onkol*. 1973; 79:304–308.
14. Bulay O, Mirvish SS, Garcia H, Pelfrene A, Gold B, Eagen M. Carcinogenicity test of six nitrosamides and a nitrosocyanamide administered orally to rats. *J Natl Cancer Inst*. 1979; 62:1523–1528. [PubMed: 286123]
15. Bartsch H, Ohshima H, Pignatelli B, Calmels S. Human exposure to endogenous N-nitroso compounds: quantitative estimates in subjects at high risk for cancer of the oral cavity, oesophagus, stomach and urinary bladder. *Cancer Surv*. 1989; 8:335–362. [PubMed: 2696584]
16. Hofmann U, Schwab M, Seefried S, Marx C, Zanger UM, Eichelbaum M, Mürdter TE. Sensitive method for the quantification of urinary pyrimidine metabolites in healthy adults by gas chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2003; 791:371–380.
17. Jiang H, Jiang J, Hu P, Hu Y. Measurement of endogenous uracil and dihydrouracil in plasma and urine of normal subjects by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002; 769:169–176.
18. van Kuilenburg AB, van Lenthe H, van Cruchten A, Kulik W. Quantification of 5,6-dihydrouracil by HPLC-electrospray tandem mass spectrometry. *Clin Chem*. 2004; 50:236–238. [PubMed: 14709662]
19. Sparidans RW, Bosch TM, Jorger M, Schellens JH, Beijnen JH. Liquid chromatography-tandem mass spectrometric assay for the analysis of uracil, 5,6-dihydrouracil and beta-ureidopropionic acid in urine for the measurement of the activities of the pyrimidine catabolic enzymes. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006; 839:45–53.
20. Solomon, JJ. DNA adducts of lactones, sultones, acylating agents and acrylic compounds. In: Hemminki, K.; Dipple, A.; Shuker, DEG.; Kadlubar, FF.; Segerbäck, D.; Bartsch, H., editors. DNA Adducts: Identification and Biological Significance. International Agency for Research on Cancer; Lyon: 1994. p. 179-198.
21. Chen L, Wang M, Villalta PW, Luo X, Feuer R, Jensen J, Hatsukami DK, Hecht SS. Quantitation of an acetaldehyde adduct in human leukocyte DNA and the effect of smoking cessation. *Chem Res Toxicol*. 2007; 20:108–113. [PubMed: 17226933]
22. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc, B*. 1995; 57:289–300.
23. National Toxicology Program. NTP Technical Report No. 495, Toxicology and Carcinogenesis Studies of Sodium Nitrite (CAS NO. 7632–00–0) Drinking Water Studies in F344/N Rats and B6C3F₁ Mice. National Toxicology Program; 2001. p. 9–26–2001
24. Svobait R, Solassol I, Pinguet F, Ivanauskas L, Bres J, Bressolle FM. HPLC with UV or mass spectrometric detection for quantifying endogenous uracil and dihydrouracil in human plasma. *Clin Chem*. 2008; 54:1463–1472. [PubMed: 18635751]
25. Mirvish SS. Formation of *N*-nitroso compounds: chemistry, kinetics, and in vivo occurrence. *Toxicol Appl Pharmacol*. 1975; 31:325–351. [PubMed: 238307]

26. Kunkel TA. Mutational specificity of depurination. *Proc Natl Acad Sci USA*. 1984; 81:1494–1498. [PubMed: 6369329]
27. Dahlmann HA, Vaidyanathan VG, Sturla SJ. Investigating the biochemical impact of DNA damage with structure-based probes: abasic sites, photodimers, alkylation adducts, and oxidative lesions. *Biochemistry*. 2009; 48:9347–9359. [PubMed: 19757831]

Highlights

- The DNA adduct 7-(2 -carboxyethyl)guanine is present in human liver.
- Nitrosation of dihydrouracil in vivo leads to elevated 7-CEGua in rat hepatic DNA.
- The hepatocarcinogen *N*-nitrosodihydrouracil can be formed in vivo in rats.
- The normal human pyrimidine metabolite dihydrouracil may be a carcinogen precursor.
- Mass spectrometry detects 7-carboxyethylguanine in human leukocyte DNA.

Fig. 1.

Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis of a hydrolysate of rat liver DNA for 7-CEGua, as its methyl ester. SRM was carried out at m/z 238-152 for 7-CEGua methyl ester, shown in the upper panels, and at m/z 243-157 for the internal standard $[^{15}\text{N}_5]$ 7-CEGua methyl ester, shown in the lower panels. (A) Chromatograms obtained from the analysis of hepatic DNA from a control animal. (B) Chromatograms obtained from the analysis of hepatic DNA from a rat exposed to DHU. (C) Chromatograms obtained from the analysis of hepatic DNA from a rat exposed to DHU + NaNO_2 .

Fig. 2. Chromatograms obtained upon LC-NSI-HRMS/MS analysis of rat hepatic DNA for 7-CEGua, as its methyl ester. The transition m/z 238 $\rightarrow$ 152.0567 was monitored for 7-CEGua methyl ester (left panel) and the transition m/z 243 $\rightarrow$ 157.0419 was monitored for the internal standard [$^{15}\text{N}_5$]7-CEGua methyl ester (right panel). The sample was from an animal exposed to DHU + NaNO_2 .

Fig. 3.

Chromatograms obtained upon LC-NSI-HRMS/MS analysis of five human leukocyte DNA samples for 7-CEGua as its methyl ester using the LTQ-Orbitrap Velos. The transition m/z 238 $\rightarrow$ 152.0567 was monitored for 7-CEGua methyl ester (upper panels) and the transition m/z 243 $\rightarrow$ 157.0419 was monitored for the internal standard $[^{15}\text{N}_5]$ 7-CEGua methyl ester (lower panels).

Scheme 1.

Structures and relationships of the compounds discussed in this study.

Table 1
Comparison of rat body weights and consumption of food and water upon treatment for two weeks (Study 1)^a

Treatment	Body weight (g, mean $\pm$ S.D.)		Consumption (mean $\pm$ S.D.)		Daily dose/rat (μ mol)
	Before treatment	After treatment	Diet (g/rat/day)	Water (ml/rat/day)	
NaNO ₂ (1500 ppm)	192.0 $\pm$ 7.0	223.5 $\pm$ 6.4	14.5 $\pm$ 0.9	18.6 $\pm$ 0.5	404 $\pm$ 12
DHU (2480 ppm)	196.4 $\pm$ 5.1	225.4 $\pm$ 7.0	14.5 $\pm$ 1.1	20.3 $\pm$ 1.3	318 $\pm$ 25
DHU + NaNO ₂ (2480+1500 ppm)	189.0 $\pm$ 6.3	216.8 $\pm$ 10.	14.5 $\pm$ 1.3	18.9 $\pm$ 0.7	318 $\pm$ 25 (DHU) 416 $\pm$ 16 (NaNO ₂)
-UPA (2870 ppm)	202.1 $\pm$ 6.9	228.6 $\pm$ 6.4	14.7 $\pm$ 0.7	18.9 $\pm$ 0.9	323 $\pm$ 15
-UPA + NaNO ₂ (2870+1500 ppm)	204.9 $\pm$ 2.7	232.6 $\pm$ 7.8	16.1 $\pm$ 1.3	19.8 $\pm$ 1.7	354 $\pm$ 29 (-UPA) 430 $\pm$ 16 (NaNO ₂)
Acrylic acid (1565 ppm)	196.0 $\pm$ 8.7	232.0 $\pm$ 8.7	15.3 $\pm$ 1.4	19.8 $\pm$ 0.9	440 $\pm$ 20
Control	193.5 $\pm$ 5.7	229.9 $\pm$ 5.5	16.3 $\pm$ 1.2	20.7 $\pm$ 1.8	

^aValues are mean $\pm$ S.D. (N=4), except acrylic acid and control (N = 6)

Table 2

Comparison of rat body weights and food and water consumption upon treatment for four weeks (Study 2)^a

Treatment	Body weight (g, mean $\pm$ S.D.)		Consumption (mean $\pm$ S.D.)		Daily dose/rat (μ mol)
	Before treatment	After treatment	Diet (g/rat/day)	Water (ml/rat/day)	
NaNO ₂ (1500 ppm)	265 $\pm$ 6	308 $\pm$ 6	15.2 $\pm$ 1.6	19.7 $\pm$ 3.8	428 $\pm$ 83
DHU (2480 ppm)	249 $\pm$ 8	300 $\pm$ 8	15.4 $\pm$ 1.4	20.4 $\pm$ 3.5	335 $\pm$ 58
DHU + NaNO ₂ (2480+1500 ppm)	266 $\pm$ 9	315 $\pm$ 7	16.8 $\pm$ 2.3	20.0 $\pm$ 3.6	365 $\pm$ 50 (DHU) 435 $\pm$ 78 (NaNO ₂)
-UPA (2870 ppm)	256 $\pm$ 13	308 $\pm$ 16	15.9 $\pm$ 1.1	21.2 $\pm$ 3.7	346 $\pm$ 60
-UPA + NaNO ₂ (2870+1500 ppm)	258 $\pm$ 9	308 $\pm$ 11	15.2 $\pm$ 0.9	19.2 $\pm$ 4.4	331 $\pm$ 20 (-UPA) 417 $\pm$ 96 (NaNO ₂)
Acrylic acid (3130 ppm) (1565 ppm)	262 $\pm$ 8	314 $\pm$ 10	15.1 $\pm$ 1.0	18.1 $\pm$ 3.7	787 $\pm$ 161
	255 $\pm$ 13	305 $\pm$ 14	14.7 $\pm$ 1.2	18.6 $\pm$ 3.9	404 $\pm$ 85
Control	263 $\pm$ 25	318 $\pm$ 32	16.7 $\pm$ 1.8	20.5 $\pm$ 3.0	

^aValues are mean $\pm$ S.D. (N=4), except control (N = 6)

Table 3

7-CEGua in hydrolysates of hepatic DNA of rats in Studies 1 and 2.

A. Study 1 (2 weeks)		B. Study 2 (4 weeks)	
Treatment	7-CEGua (fmol/ μ mol Gua) ^a	Treatment	7-CEGua (fmol/ μ mol Gua) ^a
NaNO ₂	498 $\pm$ 87	NaNO ₂	744 $\pm$ 145
DHU	445 $\pm$ 122	DHU	762 $\pm$ 52
DHU + NaNO ₂	828 $\pm$ 159 ^{b,c}	DHU + NaNO ₂	2876 $\pm$ 95 ^g
-UPA	223 $\pm$ 80	-UPA	447 $\pm$ 98
-UPA + NaNO ₂	650 $\pm$ 182 ^{d,e}	-UPA + NaNO ₂	977 $\pm$ 369 ^{h,i}
Acrylic acid	330 $\pm$ 36 ^f	Acrylic acid (1565 ppm) (3130 ppm)	809 $\pm$ 232 ^j
			1085 $\pm$ 162 ^k
None	310 $\pm$ 62	None	516 $\pm$ 98

^aValues are mean $\pm$ S.D. of N = 4 rats per group except NaNO₂, 2 weeks (3 rats), DHU + NaNO₂, 4 weeks (3 rats), acrylic acid, 2 weeks (6 rats), and controls, 2 and 4 weeks (6 rats)

^bP = 0.007 compared to NaNO₂

^cP = 0.001 compared to DHU

^dNS compared to NaNO₂

^eP = 0.0003 compared to -UPA

^fNS compared to none

^gP = 0.0003 compared to NaNO₂ or DHU

^hP = 0.04 compared to NaNO₂

ⁱP = 0.0003 compared to -UPA

^jP = 0.006 compared to none

^kP = 0.0003 compared to none