Free Rad. Res., Vol. 28, pp. 377–382
Reprints available directly from the publisher
Photocopying permitted by license only

Urine 8-Oxo-7,8-Dihydro-2′-Deoxyguanosine vs. 5-(Hydroxymethyl) Uracil as DNA Oxidation Marker in Adriamycin-Treated Patients

HENRI FAURE[a,*], MIREILLE MOUSSEAU[b], JEAN CADET[c], CATHERINE GUIMIER[a], MICHELLE TRIPIER[a], HASSAN HIDA[a] and ALAIN FAVIER[a]

[a]*G.R.E.P.O., Faculté de Médecine-Pharmacie, Domaine de la merci, F-38700 La Tronche, France;* [b]*Service d'Oncologie, C.H.U.G., BP 217, F-38043 Grenoble Cedex 9, France;* [c]*Département de Recherche Fondamentale sur la Matière Condensée – SCIB/CEA. Grenoble, F-38054 Grenoble Cedex 9 France*

Accepted by Prof. H. Sies

(Received 5 December 1997; In revised form 23 January 1998)

We measured the base 5-(hydroxymethyl) uracil (HMUra) and the nucleoside 8-oxo-7,8-dehydro-2′-deoxyguanosine (8-oxo-dGuo) in urine of adriamycin-treated cancer patients. Adriamycin has been shown to generate oxygen free radicals by various mechanisms. HMUra and 8-oxo-dGuo are two known lesions of DNA, produced by oxygen free reaction on thymine and 2′-deoxyguanosine, respectively. HMUra was measured by GC-MS/isotopic dilution and 8-oxo-dGuo by HPLC/EC, both after prepurification by semipreparative HPLC. Here we report the results of a study involving 20 cancer patients treated with flash doses of ADR. We found that urine HMUra is significantly increased (HMUra (nmol/24 h): 80.8 ± 8.44 vs. 98.7 ± 6.87; $p < 0.01$) 24 h after administration of the drug, while 8-oxo-dGuo did not show any significant variation. Urine HMUra seems to be a suitable short-term marker of DNA alterations by oxygen free radicals.

Keywords: DNA damage, 5-(hydroxymethyl) uracil, 8-oxo-7,8-dihydro-2′-deoxyguanosine, adriamycin, human cancer

INTRODUCTION

In the past, various DNA oxidative lesions have been measured in leukocytes, in blood, and in urine. However, these lesions have been measured only in few people and they have never been compared, in terms of biological sensitivity, in humans. Such comparisons could be useful to choose a good marker in situations when environmental or occupational DNA damage by free radicals is expected and has to be evaluated. These situations could be: toxic ingestion and inhalation, or exposure to ionizing radiation.

Adriamycin (ADR) is commonly used to fight cancer.[1] The reduction of the central quinone gives rise to a semiquinone free radical. Then, this radical can form superoxide radicals, while the parental ADR molecule is regenerated.[2]

*Corresponding author. Laboratoire de Biochimie C – C.H.U.G. BP 217, F-38043 Grenoble Cedex 09 France.

Flavoproteins favor the formation of semiquinone radicals by taking up electrons from NADH and NADPH and transferring them to doxorubicin. Reduction of oxygen to superoxide regenerates the parental doxorubicin molecule. This mechanism produces large amounts of •OH radicals within the cell which cause DNA damage, lesions to membranes, and to various cell structures.[3] As it generates many oxygen free radicals, mainly •OH, ADR provides an interesting model for the assessment of oxidation markers *in vivo*.

In this paper, we compare and discuss the measurement of two DNA oxidative lesions, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) and 5-(hydroxymethyl) uracil (HMUra), in 20 patients treated with adriamycin.

HMUra is formed by •OH radical attack on the methyl group of thymine.[4,5] HMUra is removed from DNA by 5-(hydroxymethyl) uracil DNA glycosylase,[6,7] eliminated from cells in blood and eventually excreted in urine. HMUra and 5-(hydroxymethyl)-2′-deoxyuridine have been used as markers of cellular DNA damage by free radicals. In a previous study involving 14 patients we have shown that ADR treatment increases HMUra excretion by 30%.[8]

Besides HMUra, 8-oxo-dGuo is one of the most abundant oxidative lesion to DNA.[9] Whereas hydroxyl radicals generate multiple products from all four bases, singlet oxygen preferentially alters 2′-deoxyguanosine, particularly through 8-hydroxylation.[10] *In vivo*, damaged nucleotides are released from nucleic acids by exonucleases or nucleotide-excision-repair. Thereafter, these nucleotides may form nucleosides after dephosphorylation. On the other hand, specific glycosylases give rise to free bases which are then excreted in urine.[9] Thus 8-oxo-dGuo has been proposed as a biomarker of oxidative DNA damage and repair, and it has been the most frequently reported marker of DNA oxidation. The base 8-oxo-7,8-dihydroguanine can be repaired by Fapy glycosylase, but it has never been used in urine as a marker of DNA oxidation and its measurement is much more difficult than 8-oxo-dGuo. For these reasons, we here compare HMUra to a more widely accepted marker of DNA oxidation *in vivo*.

MATERIAL AND METHODS

Chemicals

HMUra was from Sigma (St. Louis, MO, USA). N-(*tert.*-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) from Fluka (Mulhouse, France) was used to prepare volatile derivatives. Water for HPLC was purified with a Milli-Q system (Millipore, Milford, MA,). The internal standard for GC–MS was [1,3-$^{15}N_2$, 5-$^{2}H_2$] HMUra, synthesized from [1,3-$^{15}N_2$] uracil and [$^{2}H_2$] paraformaldehyde (Sigma).[11]

Materials

The HPLC Semipreparative Apparatus

A Kontron system (Kontron Analytic, Montigny-le-Bretonneux, France) was used. It was composed of two 420 pumps, a Kontron 460 autosampler, and a 450 data system that controlled pump flow and calculated peak areas. A Shimadzu SP6AV UV/Visible (Shimadzu Electronics, Tokyo, Japan) detector monitored the column output. For HMUra measurements semipreparative HPLC was performed with a 250 mm × 7.5 mm I.D. stainless steel column that was packed with Spherisorb ODS2 5 µm. Urine samples were eluted with water at 2.5 ml/min. After each run, the column was recycled with acetonitrile/water (60 : 40, v/v). For 8-oxo-dGuo measurements, urine samples were purified on a 250 mm × 10.0 mm I.D. stainless steel column packed with an Alltech Adsorbosphere C18 HS stationary phase. Samples were eluted with a 5 mM phosphate buffer adjusted at pH 7 and containing 2.4% acetonitrile. The pump flow was 4.4 ml/min. The components of the mobile phases were first filtered through Millipore 0.45 µm filters and degassed under vacuum. Fractions containing

HMUra or 8-oxo-dGuo were collected with a Pharmacia FRAC-100 fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Gas Chromatograph-Mass Spectrometer

Quantitative determination of HMUra was achieved with a Nermag R-10-10C Quadrupole mass spectrometer (Quad Service, Paris, France) equipped with a Nermag data system. GC separations were completed on a Delsi DI200 chromatograph (Perkin Elmer, Saint Quentin en Yvelines, France) that was equipped with a 25 m × 0.32 mm I.D. Chrompack CF Sil 5 column (Chrompack, Les Ullis, France) internally lined by a 1.2 μm film. Derivatized extracts of samples and standards were injected onto the column with an all-glass falling needle injector.

HPLC/EC Apparatus

8-oxo-dGuo was measured with a Kontron HPLC system consisting of a 422 pump, a 360 autosampler, and an ESA Coulochem II coulometer equipped with a 5011 analytical cell. Separations were performed on an Alltech Alltima C18 column (250 mm × 4.6 mm I.D.) eluted with a 20 mM phosphate buffer adjusted at pH 3 and containing 5% acetonitrile.

Other Apparatus

A Hitachi 917 autoanalyser (Boehringer-Mannheim France S.A., Meylan, France) was used to measure creatinine and uric acid in urine.

Biochemical Determinations

HMUra was determined in 24-h urine collections by GC–MS and isotopic dilution using a method that we have previously described.[12]

8-oxo-dGuo was also measured in 24-h urine collection by HPLC/EC according to a method which was published elsewhere.[13,14] Instead of using a coupled-column HPLC system, we extracted 8-oxo-dGuo from urine by using semi-preparative HPLC. An authentic standard was first injected to measure the exact retention time of 8-oxo-dGuo, 0.3 min was subtracted from the peak start time. To achieve total recovery of 8-oxo-dGuo, the time interval corresponding to the HPLC fraction of the 8-oxo-dGuo standard was increased by 0.3 min on each side. Thus all the 8-oxo-dGuo contained in urine was collected by the automatic fraction collector. For prepurification, 300 μl (150 μl × 2) of urine were injected onto the semi-preparative column and column effluent was collected for 2.5 min. Then, the collected effluent was evaporated to dryness under vacuum, and the residue was dissolved in 500 μl of a water–methanol (87/13%) mixture.

Using this technique the percentage of recovery of a 2.2 nmol/l standard solution treated as a urine sample averaged 97–99%. The limit of detection, determined as 3 times the standard deviations of ten water samples injected instead of urine, was as low as 55.1 fmol/l and the limit of quantification (10 × SD) was 165 fmol/l. Within-run precision ($n = 10$) was 4.31%, and between-run precision (10 determinations on two different days) was 5.38%.

Thiobarbituric acid reactive materials were measured as indicated by Richard *et al.*[15]

Jaffé's picric acid method[16] was used to measure urine creatinine.

Patients

Twenty patients were included in this study, all were from the Onco-Hematology Department of the Grenoble University Hospital. Seventeen were suffering from myosarcoma and uterine sarcoma, and cancer affecting different organs (breast, testicle, lung and cavum), 3 patients were suffering from hematological malignancies (2 non-Hodgkin's lymphomas and 1 plasmocytoma). All patients were included after they gave their informed consent. They were randomly selected among patients whose malignancies required ADR treatment. Procedures used in this study strictly applied the Helsinki Conference guidelines for research on human subjects.

Every patient received 30 mg adriamycin/m^2 every month, or more. ADR was associated with ifosfamide, in three patients it was associated with vincristine and dexamethasone, in one with cyclophosphamide and 5-fluorouracil, and in one with methotrexate and cisplatinum. These drugs were administered through a 2.5-h infusion, patients treated with ifosfamide or cisplatinum received also a 21 intravenous infusion. Drug infusion started at 2:00 pm and was finished by 4:30 pm.

Sample Collection

Blood Samples

Three blood samples were collected per patient in heparin containing Vacutainer® tubes (Becton Dickinson, Meylan, France). The first blood sample was taken 10–15 min before starting chemotherapy, the second sample was collected 5–6 h after the first, and the third was collected 24 h after the beginning of drug infusion.

Urine Collection

Patients collected 24-h urine the day before chemotherapy. A second 24-h urine collection was started at the end of drug infusion and stopped at the same time the following day. During collection, urine was conserved at +4°C.

Statistical Analysis

Results were analyzed using PCSM statistical software (Deltasoft, Meylan, France) run on a 386 IBM compatible PC. In blood, normally distributed variables were analyzed using a variance analysis with repeated measures, while non-normally distributed variables were tested with a Friedman test.[17] In urine, we used a paired *t*-test or a Wilcoxon *t*-test, respectively, for normal and non-normal variables. Differences of $p < 0.05$ were considered statistically significant.

TABLE I HMUra, 8-oxo-dGuo and urine data ($n = 20$; mean ± SEM)

	Before chemotherapy	After chemotherapy
24-h urine volume (l)	2.1 ± 0.29	3.6 ± 0.33**
Creatinine (mmol/24 h)	9.92 ± 0.92	11.84 ± 0.86*
Uric acid (mmol/24 h)	3.09 ± 0.26	4.37 ± 0.36**
HMUra (nmol/24 h)	80.8 ± 8.44	98.7 ± 6.87**
HMUra/creat. (nmol/mmol)	7.75 ± 0.54	8.65 ± 0.55*
8-oxo-dG (nmol/24 h)	34.4 ± 5.09	35.5 ± 4.59
8-oxo-dG/creat. (nmol/mmol)	3.77 ± 0.58	3.50 ± 0.52

Significance as compared with the level before chemotherapy: *$p < 0.05$; **$p < 0.01$.

RESULTS

Urine results are reported in Table I. Daily volume of urine increased very significantly ($p < 0.0001$). Creatinine excretion also was significantly higher ($p = 0.0180$) after chemotherapy. These two changes can be accounted for by the infusion patients received.

HMUra 24-h excretion increased significantly ($p = 0.004$) after chemotherapy and the HMUra/creatinine ratio also increased, but less significantly ($p = 0.022$). Conversely, 8-oxo-dGuo 24-h excretion and 8-oxo-dGuo/creatinine ratio showed no significant changes between the beginning and the end of chemotherapy levels. Creatinine ratios correct for renal interference in urine excretion that may occur and for muscle mass; we consider them as more reliable indexes than simple 24-h excreted amount.

Plasma TBARS increased from 2.71 ± 0.094 µmol/l (mean ± SEM) before chemotherapy to 2.75 ± 0.086 µmol/l at its end, and 2.91 ± 0.105 ($p = 0.047$) 24 h after chemotherapy.

DISCUSSION

The anticancer action of ADR is mostly due to its effect on topoisomerase II. This leads to DNA fragmentation and cell death.[1] ADR can also generate free radicals, which explains its

cardiotoxicity.[18] One-electron reduction of ADR produces a semiquinone which is a free radical. ADR semiquinone can reduce molecular oxygen to give ADR and superoxide that subsequently dismutates to H_2O_2. Thereafter, H_2O_2 and superoxide, in presence of Fe^{2+}/Fe^{3+}, can generate hydroxyl radicals by the Haber–Weiss cycle. Iron chelators decrease the ADR-mediated production of oxygen free radicals along with its cardiotoxicity in patients[19,20] without impairing the anticancerous action. ADR has not the same action on RNA because the deleterious effect of ADR requires a complex which cannot form with RNA.[21]

Our results suggest that, in the ADR model, HMUra is a suitable marker for detecting oxygen free radicals *in vivo*. Indeed, HMUra urine excretion increased by 22% after chemotherapy while 8-oxo-dGuo did not show any significant variation. Our results show that the rate of production of HMUra may be higher than that of 8-oxo-dGuo. Thymine residues in DNA may be more susceptible to oxidation than 2-deoxyguanosine, but probably not at the methyl group. Furthermore, a significant amount of the guanosine altered by hydroxylation at C8 could be released and excreted in urine as the free hydroxylated base, and would not be detected when measuring 8-oxo-dGuo alone.

In native DNA, 8-oxo-dGuo is expected to be 10-fold more abundant than 5-(hydroxymethyl)-2′-deoxyuridine. The ratios of oxidized compound to the intact, increase in the same order of magnitude when DNA is exposed to an oxidative stress such as ionizing radiation[22–24] a stress that can be compared to the action of ADR-generated free radicals. Therefore, a possible explanation for the differences we observed may be a different kinetics of release for HMUra and 8-oxo-dGuo. HMUra is repaired by a specific glycosylase, while 8-oxo-dGuo is removed by non-specific enzymes which could be less effective. However, it should be kept in mind that significant amounts of 8-oxoGua are released from DNA by Fapy glycosylase. Enzymes known to release 8-oxo-dGuo from nucleic acids are exonucleases, excision repair enzymes and perhaps some endonucleases. Being non-specific, these enzymes could be slower than HMUra glycosylase.

In vivo ADR administration results in an oxidative stress of short duration that produces a peak excretion of HMUra in urine, but it did not last long enough to increase significantly 8-oxodG by the time we measured it in urine. Such kinetic differences would explain why 8-oxodG in urine is a good marker for long-term intoxications like tobacco addiction: in these cases 8-oxodG level in DNA increases and subsequently increases its release in blood and urine.

Concerning the measurement of DNA lesions in physiological fluids, it should be noted that nucleotide pools, in cells as well as in blood, may contribute significantly to their presence in plasma and urine, when submitted to an oxidative stress. Future work should focus on determining the contribution of this production on genuine DNA repair, when bases or nucleosides are measured in blood or urine to monitor nucleic acid oxidation.

Acknowledgments

The authors thank P. -E. Colle, English teacher at the Université Joseph Fourier, for reviewing this article. HMUra and 8-oxo-dGuo determinations were run competently by Michelle Tripier, Nicole Belin and Daniel Ruffieux.

References

[1] H. G. Keizer, H. M. Pinedo, G. J. Schuurhuis and H. Joenhe (1990) Doxorubicin (Adriamycin), a critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmacology and Therapeutics* **47**, 219–231.

[2] B. K. Sinha, M. A. Trush, K. A. Kennedy and E. G. Mimnaugh (1984) Enzymatic activation and binding of adriamycin to nuclear DNA. *Cancer Research* **44**, 2892–2896.

[3] N. J. Severs, V. W. Twist and T. Powell (1991) Acute effects of Adriamycin on the macromolecular organization of the cardiac muscle cell plasma membrane. *Cardioscience* **2**(1), 35–45.

[4] C. Decarroz, J. R. Wagner, J. E. van Lier, C. Murali Krishna, P. Riesz and J. Cadet (1986) Sensitized photooxidation of thymidine by 2-methyl-1,4-naphtoquinone. Characterization of stable photoproducts. *International Journal of Radiations in Biology* **50**(3), 491–505.

[5] B. N. Ames and R. L. Saul (1986) Oxidative DNA damage as related to cancer and aging. In, *Genetic Toxicology of Environmental Chemicals, Part A, Basic Principles and Mechanisms of action*. Alan R. Liss Inc., New York, pp. 11–26.

[6] M. C. Hollstein, P. Brooks, S. Linn and B. N. Ames (1984) Hydroxymethyluracil DNA glycosylase in mammalian cells. *Proceedings of the National Academy of Science of USA* **81**, 4003–4007.

[7] Z. Djuric, C. K. Everett and D. Luongo (1993) Toxicity, single-strand breaks, and 5-hydroxymethyl 2'-deoxyuridine formation in human breast epithelial cells treated with hydrogen peroxide. *Free Radical Biology & Medicine* **14**, 541–547.

[8] H. Faure, C. Coudray, M. Mousseau, V. Ducros, T. Douki, F. Bianchini, J. Cadet and A. Favier, (1996) 5-Hydroxymethyluracil excretion, plasma TBARs and plasma antioxidant vitamins in adriamycin-treated patients. *Free Radical Biology & Medicine* **20**(7), 979–983.

[9] S. Loft, A. Fisher-Nielsen, I. B. Jeding, K. Vistisen and H. E. Poulsen, (1993) 8-Hydroxydeoxyguanosine as a urinary biomarker of oxidative damage. *Journal of Toxicology and Environmental Health* **40**, 391–404.

[10] B. Epe (1991) Genotoxicity of singlet oxygen. *Chemical and Biological Interactions* **80**, 239–260.

[11] R. E. Cline, R. M. Fink and K. Fink (1959) Synthesis of 5-substituted pyrimidines via formaldehyde addition. *American Chemistry Society* **81**, 2521.

[12] H. Faure, F. Incardona, C. Boujet, J. Cadet, V. Ducros and A. Favier (1993) Gas chromatographic-mass spectrometric determination of 5-hydroxymethyluracil in human urine by stable isotope dilution. *Journal of Chromatography* **616**, 1–7.

[13] S. Loft, K. Vistisen, M. Ewertz, A. Tjonneland, K. Overvad and H. -E. Poulsen (1992) Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans, influence of smoking, gender and body mass index. *Carcinogenesis* **13**(12), 2241–2241.

[14] C. Tagesson, N. Kallberg and P. Leanderson (1992) Determination of urinary 8-hydroxydeoxyguanosine by coupled column HPLC with electrochemical detection, a noninvasive assay for *in vivo* oxidative DNA damage in humans. *Toxicological Methods* **1**(4), 242–251.

[15] M.-J. Richard, B. Portal, J. Meo, C. Coudray, A. Hadjian and A. Favier (1992) Malondialdehyde kit evaluated for determining plasma and lipoprotein fractions that react with thiobarbituric acid. *Clinical Chemistry* **38**(5), 704–709.

[16] H. Bartels, N. Böhmera and C. Heterli (1972) Serumkreatininbestimmung ohne enteiweissen (Serum creatinine determination without protein precipitation). *Clinica Chimica Acta* **37**, 193–197.

[17] B. J. Winer, D. Brown and K. M. Michels (1991) *Statistical principles in experimental design. Third edition*. Mac Graw-Hill Inc., New York.

[18] F. Monti, F. Prosperi, R. Supino and G. Bottiroli (1995) Free radical-dependant DNA lesions are involved in the delayed cardiotoxicity induced by adriamycin in the rat. *Anticancer Research* **15**(1), 193–197.

[19] C. Hershko, G. Link, M. Tzahor and A. Pinson (1993) The role of iron and iron chelators in anthracycline cardiotoxicity. *Leukemia and Lymphoma* **11**(3,4), 207–214.

[20] J. L. Speyer, M. D. Green, E. Kramer, M. Rey, J. Sanger, C. Ward, N. Dubin, V. Ferrans, P. Stery, A. Zeleniuch-Jacquotte, J. Wernz, F. Feit, W. Slater, R. Blum and F. Muggia (1988) Protective effect of the bispiperazinedione ICRF-187 against Doxorubicin-induced cardiac toxicity in women with advanced breast cancer. *New England Journal of Medicine* **319** (September 22), 745–752.

[21] E. Feinstein, E. Canaani and L. M. Weiner (1993) Dependence of nucleic acid degradation on *in situ* free-radical production by Adriamycin. *Biochemistry* **32**, 13156–13161.

[22] D. S. Bergtold, M. C. Simic, H. Alessio and R. G. Cutler, (1988) Urine biomarkers for oxidative DNA damage. In, *Oxygen Radicals in Biology and Medicine* (Eds. M. G. Simic, K. A. Talor and J. F. Ward) Plenum, New York, pp. 483–489.

[23] D. S. Bergtold, C. D. Berg and M. C. Simic (1990) Urinary biomarkers in radiation therapy of cancer. *Advance in Experimental Medicine and Biology* **264**, 311–316.

[24] M. Dizdaroglu (1992) Oxidative damage to DNA in mammalian chromatin. *Mutation Research* **275**, 331–342.