Letter

Identification of dihydropyrazine-glutathione adducts

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ABSTRACT — Dihydropyrazines (DHPs) are glycation intermediates generated both *in vivo* and in food. DHPs can lead to the formation of a variety of different radical species, which can lead to DNA damage and enzyme inhibition. In addition, the presence of DHPs can lead to a decrease in cellular glutathione (GSH) levels, and induce the expression of antioxidant genes. In this study, the products resulting from the reaction of DHP with GSH have been analyzed in detail, with some of the products being separated by reversed-phase HPLC. The structures of the isolated DHP-GSH adducts were determined by FAB-MS and NMR analyses. These data suggested that the reaction of DHP with a thiol moiety could be involved in oxidative stress, because an increase in the amount of DHP-GSH adducts would result in a decrease in the cellular GSH levels.

Key words: Dihydropyrazine, Glutathione, Adduct, Oxidative stress, FAB-MS, NMR

INTRODUCTION

Dihydropyrazines (DHPs), which are formed by nonenzymatic glycation, have been reported to generate various radicals and reactive oxygen species (ROS) that are capable of causing DNA strand cleavage *in vitro* (Yamaguchi *et al.*, 1996). DHPs are presumed to be precursors of pyrazines, which can have an adverse impact on human health. Many pyrazine derivatives, which are produced as the metabolites of DHPs, have been found in human urine (Zlatkis *et al.*, 1973). 5-Aminolevulinic acid (ALA) is a heme precursor that accumulates in lead poisoning and inborn porphyrias, and the condensation of two molecules of ALA leads to the formation of the DHP derivative, 3,6-dihydropyrazine-2,5-dipropanoic acid, which has been reported to exhibit DNA strand-cleaving activity *in vitro* (Teixeira *et al.*, 2001). Despite concerns surrounding the effects of DHPs on human health, there is very little evidence in the literature regarding a causal relationship between DHP and disease. We previously reported that DHP causes greater growth inhibition in GSH-deficient bacteria than it does in GSH-sufficient bacteria (Takechi *et al.*, 2011), and we also observed a significant decrease in the ratio of intracellular reduced glutathione and oxidized glutathione (GSH/GSSG) in DHP-exposed cultured

cell (Ishida *et al.*, 2012). In the current study, we have identified novel DHP-GSH adducts from the reaction products of DHP with GSH. The findings of this study suggest that DHP is effectively enforcing cellular stress by the depletion of cellular GSH levels.

MATERIALS AND METHODS

General experimental procedures

¹H and ¹³C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AVANCE spectrometer (Bruker, Billerica, MA, USA) at 298 K using a DCH Cryo-probe. The spectra were measured in CD₃OD in all cases, and the residual solvent peak (δ 3.30 ppm) was used as an internal reference standard for reporting the chemical shifts. ¹H-¹H COSY (correlation spectroscopy), phase sensitive nuclear overhauser enhancement spectroscopy (NOESY), ¹H-detected heteronuclear single-bond quantum correlation (HSQC) and ¹H-detected heteronuclear multiple bond correlation (HMBC) experiments were carried out on the same instrument. Fast atom bombardment-mass spectrometry (FAB-MS) experiments using *m*-nitrobenzyl alcohol as a matrix were conducted on a JEOL JMS-HX110 double-focusing model (JEOL, Tokyo, Japan) equipped with a FAB ion source interfaced

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with a JEOL JMA-DA 700 data system. High-resolution mass spectrometry (HRMS) experiments were obtained using the same mass spectrometer.

Synthesis of DHP derivatives

3-Hydro-2,2,5,6-tetramethylpyrazine (Me-DHP-3) and Cyclohexyl-DHP (Fig. 1), as well as a mixture of the two isomers of 2,3,5,6,7,8-hexahydroquinoxaline (*endo*-type) and 1,2,3,5,6,7-hexahydroquinoxaline (*exo*-type), were prepared using a previously reported procedure (Yamaguchi *et al.*, 1998).

Reaction and isolation procedure of Adducts P1, P2, P3

A reaction mixture containing 50 mM DHP and 50 mM GSH was incubated in 0.1 M sodium phosphate buffer (pH 7) at 37°C for 24 hr, and the resulting reaction products were separated by reversed-phase HPLC using a Chromolith SemiPrep RP-18e column (10 × 100 mm; Merck, Darmstadt, Germany), which was equilibrated with a mobile phase consisting of 5% methanol in an aqueous solution of 0.1% trifluoroacetic acid at a flow rate of 3 mL/min. The elution profiles were monitored by absorbance at 210 nm (Fig. 2 A).

(Z)-2-amino-5-((1-((carboxymethyl)amino)-1-oxo-3-(((3,5,5-trimethyl-5,6-dihydropyrazin-2(1H)-ylidene)methyl)thio)propan-2-yl)amino)-5-oxopentanoic acid (P1)

HRMS-FAB-MS in the positive ionization mode gave m/z : 444.1916 (Calcd for $C_{18}H_{30}N_5O_6S$ $[M+H]^+$:

444.1917). 1H NMR: δ 7.31 (s, 1H), 4.75 (dd, $J = 8.1, 5.3$ Hz, 1H), 3.92 (dd, $J = 26.6, 17.7$ Hz, 2H), 3.68 (d, $J = 6.7$ Hz, 1H), 3.54 (dd, $J = 14.1, 5.3$ Hz, 1H, Cys- β) & 3.30*¹, 3.47 (s, 2H), 2.54 (m, 2H), 2.53 (s, 3H), 2.14 (m, 2H), 1.24 (s, 6H). ^{13}C NMR: δ 174.9 (CO₂), 173.3 (CO), 172.8 (CO₂), 172.1 (CO), 167.2 (C)*², 132.0 (CH), 131.86 (C), 54.9 (CH), 54.82 (CH₂), 54.76 (CH), 48.4 (C)*², 42.0 (CH₂), 37.2 (CH₂)*, 32.8 (CH₂), 27.6 (CH₂), 26.0 (CH₃), 18.5 (CH₃). *¹One of the germinal β -methylene protons of cysteine was concealed behind a broad solvent signal at δ 3.30 ppm, but its $^1J_{H/C}$ correlation with the corresponding β -carbon of cysteine was observed in the HSQC spectrum. *²Two quaternary carbons were not observed in the ^{13}C NMR spectrum, but the HMBC spectrum clearly showed $^3J_{H/C}$ long range correlations from δ_H 7.31 and 3.47 ppm to δ_C 167.2 ppm, as well as $^2J_{H/C}$ correlations from δ_H 1.24 and 3.47 ppm to δ_C 48.4 ppm.

(Z)-2-amino-5-((1-((carboxymethyl)amino)-1-oxo-3-(((3,5,5-trimethyl-5,6-dihydropyrazin-2(1H)-ylidene)methyl)thio)propan-2-yl)amino)-5-oxopentanoic acid (P2)

HRMS-FAB-MS in the positive ionization mode gave m/z : 444.1917 (Calcd for $C_{18}H_{30}N_5O_6S$ $[M+H]^+$: 444.1917). 1H NMR: δ 7.25 (s, 1H), 4.74 (dd, $J = 7.8, 5.8$ Hz, 1H), 3.96 (dd, $J = 27.8, 17.7$ Hz, 2H), 3.95 (d, $J = 5.7$ Hz, 1H), 3.52 (dd, $J = 14.1, 5.8$ Hz, 1H, Cys- β) & 3.29*, 3.08 (s, 2H), 2.56 (dd, $J = 14.0, 7.3$ Hz, 2H), 2.48 (s, 3H), 2.17 (m, 2H), 1.40 (s, 6H). ^{13}C NMR: δ 174.4 (CO), 172.6 (CO₂), 172.0 (CO₂), 171.9 (CO), 166.3 (C), 132.1 (C), 130.1 (CH), 56.8 (C), 54.7 (CH), 53.8 (CH), 50.1 (CH₂),

Fig. 1. Chemical structures of the DHP derivatives used in this Study.

The adducts of dihydropyrazine and glutathione

Fig. 2. Analysis of the DHP-GSH conjugates. A, HPLC profiles of the reaction mixtures of Me-DHP-3 (left) and cyclohexyl-DHP (right) with GSH. The reaction and HPLC conditions are described in the Materials and Methods section. B, FAB-MS chromatograms for peaks P1, P2 and P3. Peaks P1, P2 and P3 gave pseudo-molecular ion peaks corresponding to $[M+H]^+$ with m/z values of 444.1916, 444.1917 and 442.1767, respectively.

41.8 (CH₂), 37.0(CH₂)*, 32.4 (CH₂), 27.1 (CH₂), 25.1 (CH₃ × 2), 18.7 (CH₃). *The same as described for P1.

2-amino-5-((1-((carboxymethyl)amino)-3-((2,3,4,6,7,8-hexahydroquinoxalin-5-yl)thio)-1-oxopropan-2-yl)amino)-5-oxopentanoic acid (P3)

HRMS-FAB-MS in the positive ionization mode gave *m/z*: 442.1767 (Calcd for C₁₈H₂₈N₅O₆S [M+H]⁺: 442.1760). ¹H NMR: δ 4.63 (dd, *J* = 7.7, 6.3 Hz, 1H), 3.98(dd, *J* = 5.7 Hz, 1H), 3.93 (dd, *J* = 20.4, 6.0 Hz, 2H), 3.68 (t, *J* = 4.1 Hz, 1H), 3.51 (dd, *J* = 13.9, 6.3 Hz, 1H, Cys-β), 3.27 (dd, *J* = 13.9, 7.9 Hz, 1H, Cys-β), 3.29 (2H)*, 2.82 (m, 2H), 2.78 (m, 2H), 2.57 (t, *J* = 7.4 Hz, 2H), 2.18 (hept, *J* = 6.5 Hz, 2H), 2.01 (quint, *J* = 6.2 Hz, 2H). ¹³C NMR: δ 174.3 (CO), 172.7 (CO₂), 171.9 (CO₂), 171.8 (CO), 167.2 (C), 144.2 (C), 131.4 (C), 54.3 (CH), 53.5 (CH), 44.6 (CH₂), 41.8 (CH₂), 39.1 (CH₂)*, 33.0 (CH₂), 32.3 (CH₂), 30.6 (CH₂), 29.4 (CH₂), 27.0 (CH₂), 22.4 (CH₂). *The same as described for P1.

RESULTS AND DISCUSSION

We recently reported that DHP derivatives caused decrease in the ratio of GSH/GSSG in human hepatoma HepG2 cells (Ishida *et al.*, 2012). Based on this result, we decided to investigate the nature of the interaction between DHP and GSH, which is the most abundant intercellular thiol compound in eukaryotic organisms. Two DHP derivatives were used for this study, including Me-DHP-3 and Cyclohexyl-DHP (Fig. 1). To allow for the detection of the corresponding DHP-GSH conjugates, 50 mM solutions of the DHP derivatives were incubated with 50 mM GSH in 0.1 M sodium phosphate buffer (pH 7) at 37°C for 24 hr. The resultant products were analyzed by HPLC and the elution profiles were monitored by absorbance at 210 nm. As shown in Fig. 2A, the elution profiles revealed the loss of GSH with a concomitant increase in GSSG. Moreover, the reactions with Me-DHP-3 (mw: 138.21) and cyclohexyl-DHP (mw: 136.19) with GSH (mw: 307.33) led to the formation of new peaks with larger retention times than that of GSSG (P1 and P2, or P3, respectively). Subsequent FAB-MS analysis revealed that P1 and P2 gave similar [M+H]⁺ peaks with *m/z* values of 444.1917 and 444.1617, respectively, and that P3 gave an [M+H]⁺ peak with *m/z* value of 442.1767 (Fig. 2B). Peaks P1 and P2 therefore corresponded to the addition of Me-DHP-3 to GSH, with the two peaks representing different isomeric forms, whereas P3 corresponded to the addition of cyclohexyl-DHP to GSH. NMR analysis (Fig. 3) of the adducts formed

between Me-DHP-3 and GSH revealed the disappearance of one of the two imino methyl signal belonging to the DHP moiety. The HSQC spectra of these adducts revealed the appearance of new olefinic ¹J_{H/C} correlations from δ_H 7.31 to δ_C 132.0 ppm for P1, and δ_H 7.25 to δ_C 130.1 ppm for P2. Most notably, the HMBC spectrum of P2 revealed that presence of two ³J_{H/C} correlations from the *exo*-methyne proton at δ_H 7.25 ppm to the β-carbon of the cysteine moiety of GSH at δ_C 37.0 ppm, as well as a carbon belonging to the DHP moiety at δ_C 132.1 ppm. Furthermore, HMBC analysis of P3 revealed a reverse ³J_{H/C} correlation between the β-proton of the cysteine moiety at δ_H 3.51 ppm and a carbon belonging to the cyclohexyl moiety of Cyclohexyl-DHP at δ_C 144.2 ppm. These ³J_{H/C} correlation results therefore provided direct evidence of the formation of a new C-S bond between the thiol group of the cysteine residue and the *exo*-methyl carbon of the DHP derivative (Fig. 1). The highly reactive nature of the *exo*-methylene group of DHP has been attributed to its latent enamine functionality, and this result has been confirmed both experimentally and theoretically (Ito *et al.*, 2010). The low energy distribution of the highest occupied molecular orbital (HOMO) on the *exo*-methylene group of the DHP moiety would allow it undergo nucleophilic addition reactions with various biological thiols, including cysteine and GSH. Cyclohexyl-DHP can also participate in an intramolecular equilibrium to give cyclohexenyl-DHP (Takechi *et al.*, 2009). Based on these data, the chemical structures of these products were determined to be those shown in Fig. 4.

In this study, we have investigated the reaction of several DHP derivatives with GSH and analyzed the resultant products. DHP can be readily oxidized under mild conditions and reaches an unstable state when it donates an electron to another species, such as oxygen dissolved in the system, which can lead to the formation of a superoxide anion (Takeda *et al.*, 2005). DHP was reacted GSH, which is a cysteine-containing tripeptide, and the reaction was observed analytically. The reaction of DHP with GSH led to the rapid oxidation of GSH to give multiple products, which were detected by HPLC analysis (Fig. 2A). Structural analysis of these products suggested that the DHP-GSH adducts were produced by an oxidative dehydrogenation reaction.

GSH, which acts as an antioxidant, is one of the most abundant low-molecular weight thiols in mammalian cells (Bhattacharya *et al.*, 1955). Given that GSH is converted to GSSG following its reaction with ROS, and GSH depletion results in elevated cytotoxicity (Mitchell and Russo, 1987). In our previous study, DHP caused growth inhibition of GSH-deficient bacteria (Takechi *et*

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Fig. 3. Significant HMBC correlations for P1, P2 and P3.

Fig. 4. Structures of the DHP-GSH Conjugates. These structures were deduced from peaks P1, P2 and P3, as described in the Materials and Methods section.

al., 2011), and DHP also led to a decrease in the ratio of intracellular GSH/GSSG in cultured cells (Ishida *et al.*, 2012). In addition, the exposure of cells to DHP led to the induction of antioxidant gene expression via the activation of the Nrf2-ARE pathway (Ishida *et al.*, 2014).

In conclusion, the results of this study provides further evidence that DHP, as a generator of ROS, oxidizes GSH to give the corresponding DHP-GSH adducts, which results in the depletion of cellular GSH levels. These results therefore suggest that DHP could play a significant role in disrupting metabolic processes in cells, such as disorders associated with oxidative-antioxidative balance.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Bhattacharya, S.K., Robson, J.S. and Stewart, C.P. (1955): The determination of glutathione in blood and tissues. *Biochem. J.*, **60**, 696-702.
- Ishida, T., Takechi, S. and Yamaguchi, T. (2012): Possible involvement of glutathione balance disruption in dihydropyrazine-induced cytotoxicity on human hepatoma HepG2 cells. *J. Toxicol. Sci.*, **37**, 1065-1069.
- Ishida, T., Yamaguchi, T. and Takechi, S. (2014): The effect of dihydropyrazines on human hepatoma HepG2 cells: a comparative study using 2,3-dihydro-5,6-dimethylpyrazine and 3-hydro-2,2,5,6-tetramethylpyrazine. *J. Toxicol. Sci.*, **39**, 601-608.
- Ito, S., Hirano, T., Sugimoto, A., Kagechika, H., Takechi, S. and Yamaguchi, T. (2010): Latent enamine functionality of 5-Methyl-2,3-dihydropyrazines. *Chem. Pharm. Bull.*, **58**, 922-927.
- Mitchell, J.B. and Russo, A. (1987): The role of glutathione in radiation and drug induced cytotoxicity. *Br. J. Cancer (Suppl.)*, **55**, 96-104.
- Takechi, S., Ito, S., Kashige, N., Ishida, T. and Yamaguchi, T. (2011): Glutathione Depression by dihydropyrazine derivative. *J. Toxicol. Sci.*, **36**, 231-235.
- Takechi, S., Nakahara, K., Adachi, M. and Yamaguchi, T. (2009): Oxidative stress induced by a dihydropyrazine derivative. *Biol. Pharm. Bull.*, **32**, 186-189.
- Takeda, O., Takechi, S., Katoh, T. and Yamaguchi, T. (2005): The Role of dihydropyrazines in accelerated death of *Escherichia coli* on addition of copper(II). *Biol. Pharm. Bull.*, **28**, 1161-1164.
- Teixeira, P.C., Onuki, J., Medeiros, M.H., Dörnemann, D. and Di Mascio, P. (2001): DNA damage by 3,6-dihydropyrazine-2,5-dipropionic acid, the cyclic dimerization product of 5-aminolevulinic acid. *Biol. Chem.*, **382**, 913-918.
- Yamaguchi, T., Kashige, N., Mishiro, N., Miake, F. and Watanabe, K. (1996): Dihydropyrazine derivatives as a new type of DNA strand breaking agent. *Biol. Pharm. Bull.*, **19**, 1261-1265.
- Yamaguchi, T., Matsumoto, S. and Watanabe, K. (1998): Generation of free radicals from dihydropyrazines with DNA strand-breakage activity. *Tetrahedron Lett.*, **39**, 8311-8312.
- Zlatkis, A., Bertsch, W., Lichtenstein, H.A., Tishbee, A., Shunbo, F., Liebich, H.M., Coscia, A.M. and Fleischer, N. (1973): Profile of volatile metabolites in urine by gas chromatography-mass spectrometry. *Anal. Chem.*, **45**, 763-767.