FULL PAPER

Dalton
www.rsc.org/dalton

Inhibition of *Thermus thermophilus* HB8 thioredoxin activity by platinum(II)†

Masahiro Kato,[a] Hitoshi Yamamoto,[a,c] Taka-aki Okamura,[a] Nobuko Maoka,[c] Ryoji Masui,[b,c] Seiki Kuramitsu[b,c] and Norikazu Ueyama*[a]

[a] *Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka, 560-0043, Japan. E-mail: ueyama@chem.sci.osaka-u.ac.jp; Fax: +81-6-6850-5474; Tel: +81-6-6850-5449*

[b] *Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka, 560-0043, Japan*

[c] *the RIKEN Harima Institute/SPring-8, 1-1-1 Koto, Mikazuki-cho, Sayo-gun, Hyogo, 679-5148, Japan*

Received 21st December 2004, Accepted 27th January 2005
First published as an Advance Article on the web 16th February 2005

A 1 : 1 thioredoxin–Pt(bpy) complex **1** was prepared by adding [Pt(bpy)(en)]Cl_2 (bpy = 2,2′-bipyridine, en = ethylenediamine) to *Thermus thermophilus* HB8 thioredoxin in pH 8 phosphate buffer. Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) and UV spectra of **1** indicate the formation of Pt(bpy)(cys-Ala-Pro-cys-containing peptide fragment). These findings suggest that the $Pt(bpy)^{2+}$ unit binds to the active site of thioredoxin. The thioredoxin–platinum complex has no catalytic activity for the reduction of glutathione disulfide in the presence of NADPH and thioredoxin reductase, so that the platinum complex functions as an inhibitor.

Introduction

Thioredoxins are small dithiol oxidoreductases with a Cys(1)-X-Y-Cys(2) sequence in the active center. In many species, these enzymes have a similar three-dimensional structure referred to as the "thioredoxin fold" in X-ray crystal structure and NMR solution structure studies. Recent studies have shown that thioredoxin is overexpressed in tumors compared with levels in corresponding normal tissue.[1–8] The sensitivity of adult T-cell leukemia cell lines to doxorubicin is lowest in those cell lines with the highest levels of thioredoxin.[9] On the other hand, human hepatoma cells with increased thioredoxin show a decreased sensitivity to cell killing by *cis*-diamminedichloroplatinum(II) (cisplatin) but not by doxorubicin or mitomycin C.[10] It has also been reported that bladder and prostate cancer cell lines resistant to cisplatin have a 4–6 fold increase in levels of thioredoxin,[11] and gastric and colon cancer cell lines resistant to cisplatin show an increase by up to 2.5-fold. In addition, it has been reported that there is a correlation between cisplatin resistance and thioredoxin levels in some other cancer cell lines.[3,12] This resistance to cisplatin could be reversed by lowering thioredoxin levels, and the sensitivity of cancer cell lines to doxorubicin, mitomycin C, etoposide and H_2O_2 and UV increases. Consequently, thioredoxin causes resistance to chemotherapy by trapping the antitumor agent such as cisplatin. This finding is very interesting in that the inhibitor of thioredoxin activity inhibits the trapping of the antitumor agent and becomes a novel and rational target for antitumor agent design.

Thus far, 1-methylhydroxypropyl 2-imidazolyl disulfide (PX-12), which binds to the Cys73 residue,[13] and naphthoquinone and benzoquinone derivatives have been synthesized as inhibitors for thioredoxin.[14–16] In addition, cadmium is used as a metal inhibitor, and it has been suggested that Cd^{2+} inhibits thioredoxin activity by binding at the Cys32 in the active site and by interacting with the neighboring Asp26.[17] In thioredoxin reductase as well as in thioredoxin, metal compounds such as gold[18] and platinum[19] and metal ions such as Hg^{2+} and Cu^{2+} are used as the inhibitor.[16] On the basis of the absorption spectra, it is predicted that (2,2′:6′,2′-terpyridine)platinum(II) complexes modified Cys52 in the active site and inhibit the activity of the trypanothione reductase.[20] However, the details of the interacting site between platinum compounds and thioredoxin are unknown. In this report, we present the introduction of a platinum(II) complex to *Thermus thermophilus* HB8 thioredoxin, the binding site of the platinum(II) complex, and inhibition of thioredoxin activity in the resulting thioredoxin–platinum(II) complex.

Results and discussion

Preparation and purification of the thioredoxin–platinum complex 1

Thioredoxin was obtained by overexpression in *Escherichia coli* of the gene cloned in *T. thermophilus* HB8 and purified. After reduction by dithiothreitol and dialysis, thioredoxin was mixed with 10 equivalents of [Pt(bpy)(en)]Cl_2 (bpy = 2,2′-bipyridine, en = ethylenediamine) aqueous solution in 0.1 M phosphate buffer (pH 8). The mixture solution was incubated at 37 °C for 3 days. Fig. 1 shows the matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) spectra of thioredoxin solution before and after incubating with the platinum complex. In the mass spectrum before the mixing with the platinum complex, a clean peak assignable to thioredoxin was observed at m/z 12330, while after the mixing, a new peak was detected at m/z 12679. The new peak is assigned to the thioredoxin–platinum complex **1**. Increase of one $Pt(bpy)^{2+}$ unit (m/z 351) and decrease of $2H^+$ indicates the formation of Pt(bpy)–thioredoxin. This result suggests that chelating ethylenediamine is removed from the platinum(II) complex and that two sites of thioredoxin bind the platinum(II).

The solution after incubating for three days is a mixture of **1** and thioredoxin, as shown in Fig. 1. Subsequently, **1** was purified by 6% native-polyacrylamide gel electrophoresis (PAGE). Fig. 2 shows the electrophoregram of thioredoxin and pre-purified solution and purified solution. In the pre-purified solution, two

† Electronic supplementary information (ESI) available: MALDI-TOF MS spectra (Figs. S1, S3 and S4) and UV-visible spectrum (Fig. S2). See http://www.rsc.org/suppdata/dt/b4/b419119f/

DOI: 10.1039/b419119f

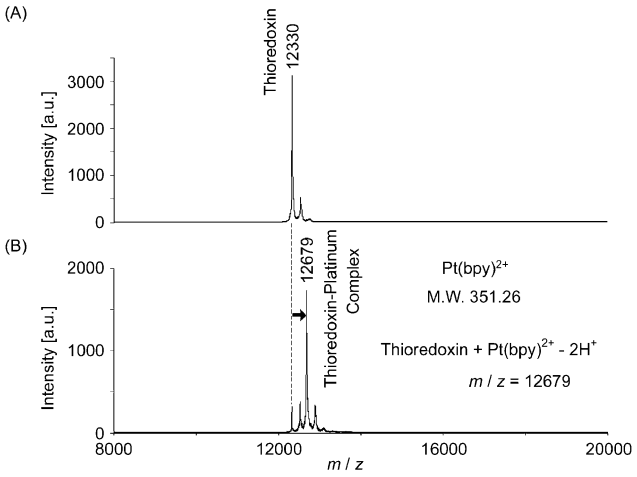

Fig. 1 MALDI-TOF MS spectra of (A) *T. thermophilus* HB8 thioredoxin and (B) after mixing with $Pt(bpy)(en)^{2+}$.

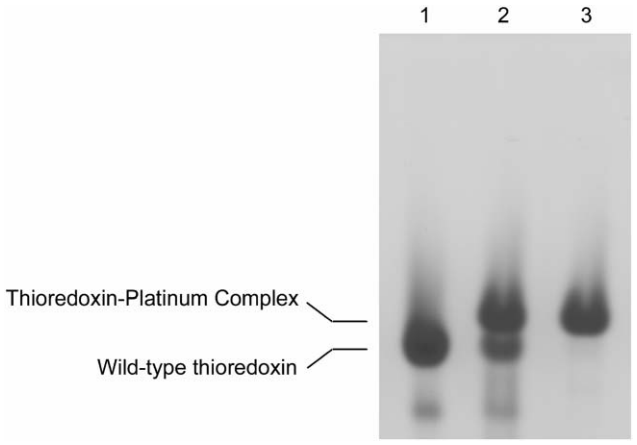

Fig. 2 Purification of **1** by 6% native-PAGE. Lane 1: Wild-type thioredoxin. Lane 2: The reaction solution after 3 days prior to pre-purification. Lane 3: After purification.

bands are detected, and one of them is detected at the same position as thioredoxin. On the other hand, in the purified solution, the band of thioredoxin disappears and only one band is detected. This finding suggests that **1** is highly purified. In addition, the MALDI-TOF MS spectrum of the purified **1** shows the disappearance of the peak (*m/z* 12330) assigned to thioredoxin (Fig. S1, ESI†). Consequently, these results suggest that **1** does not exist in solution as an equilibrium state and that the platinum complex binds strongly to thioredoxin.

UV-visible and CD spectra of 1

Fig. 3 shows the UV-visible spectra of thioredoxin, $Pt(bpy)(en)^{2+}$ and **1**. The UV-visible spectrum of **1** exhibits a broad absorption band with medium intensity at 433 nm compared with that of thioredoxin. This lowest energy absorption band is observed in $Pt(bpy)(SAr)_2$ (Ar = benzenethiolate derivatives) complexes[21,22] and [Pt(bpy)(Z-cys-Pro-Leu-cys-OMe)] complex (Fig. S2, ESI†) and assigned as the charge transfer from the HOMO of thiolate(π)/S(p)/Pt(d) to the LUMO of bipyridine(π*) character.[22] Thus, the detection of the new absorption band in **1** suggests that platinum(II) binds to sulfur, thereby forming a Pt–S bond.

To confirm the formation of the bond between platinum and the sulfur of cysteine, thioredoxin in which cysteine is alkylated by iodoacetoamide was incubated with $Pt(bpy)(en)^{2+}$ at 37 °C. As a result, a new peak assigned to **1**, as shown in Fig. 1, is undetectable (Fig. S3, ESI†). This finding supports that platinum complex binds to the sulfur of cysteine, as described above.

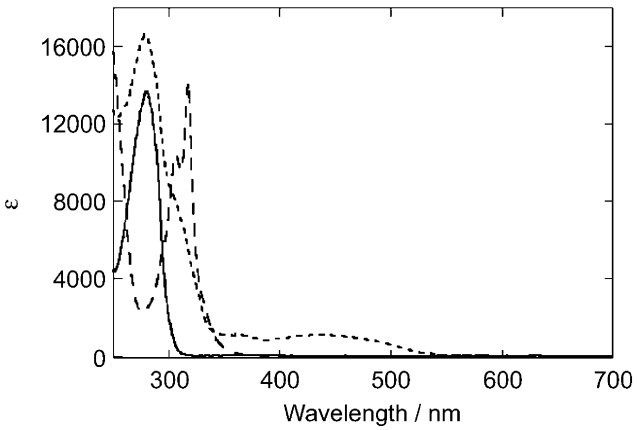

Fig. 3 UV-visible spectra of thioredoxin (—), $Pt(bpy)(en)^{2+}$ (---) and **1** (···) in pH phosphate buffer at 30 °C.

Fig. 4 shows the CD spectra of thioredoxin and **1**. The difference spectrum between them shows the decrease of the band in the vicinity of 218 nm in **1**. This band at 218 nm is assigned to the β-strand. Some X-ray structures[23–25] and NMR solution structures[26–29] of thioredoxins have been previously reported. In general, thioredoxin derivatives have a structure denoted as the 'thioredoxin fold'. Thioredoxin consists of a five-stranded β-sheet at the center of the protein, and surrounding this four α-helices and a helical turn. Therefore, the introduction of platinum complex into thioredoxin causes a small change of protein structure and somewhat disrupts the β strands in the center of protein. However, the change of the CD spectra by the introduction of platinum complex is small, and most of the structure is retained and not denatured.

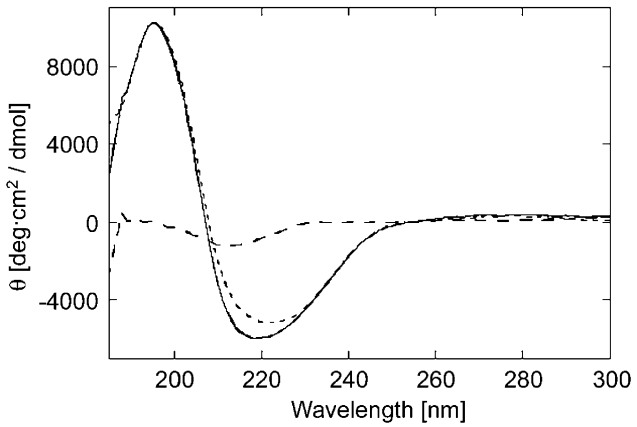

Fig. 4 CD spectra of thioredoxin (—), **1** (···) and the difference spectrum (---) in pH 7 phosphate buffer.

Digestion of 1 and MALDI-TOF MS spectrum of the digested peptides

In order to determine the binding site of platinum(II) in thioredoxin, the MALDI-TOF MS spectrum of peptide fragments of **1** digested by chymotrypsin was measured. The result and the amino acid sequence in *T. thermophilus* HB8 thioredoxin are shown in Fig. 5. The spectrum shows the two large peaks with the isotope pattern of platinum. In view of the amino acid sequence, the peaks at *m/z* 898.194 and 1267.371 are assigned as [Pt(bpy)(*cys*-Ala-Pro-*cys*-Arg) + H^+] (calc. *m/z* 898.246) and [Pt(bpy)(Ala-Glu-Trp- *cys*-Ala-Pro-*cys*-Arg) − H_2O + H^+] (calc. *m/z* 1267.371), respectively. In addition, the isotope patterns of platinum were consistent with the simulation and support the assignment of each platinum–peptide complex.

Fig. 6 and S4 (ESI†) show the LIFT TOF/TOF MS spectra obtained from [Pt(bpy)(*cys*-Ala-Pro-*cys*-Arg) + H^+]

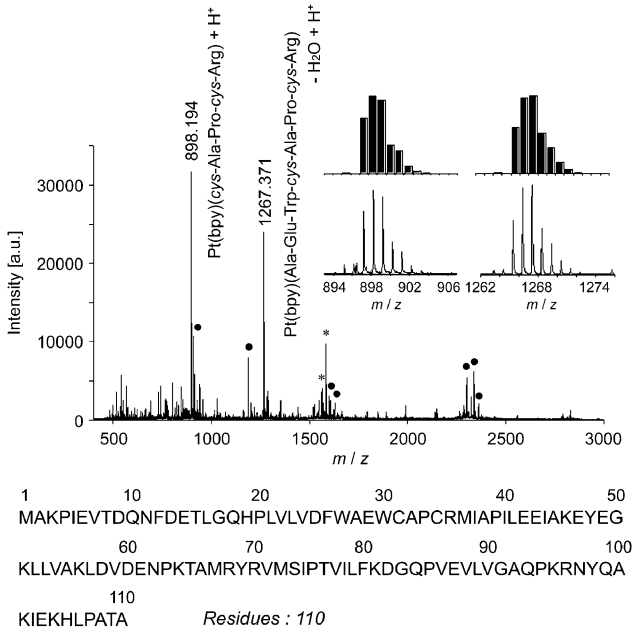

Fig. 5 MALDI-TOF MS spectrum of **1** digested by chymotrypsin (*: chymotrypsin fragment; ●: thioredoxin fragment) and the amino acid sequence of *T. thermophilus* HB8 thioredoxin.

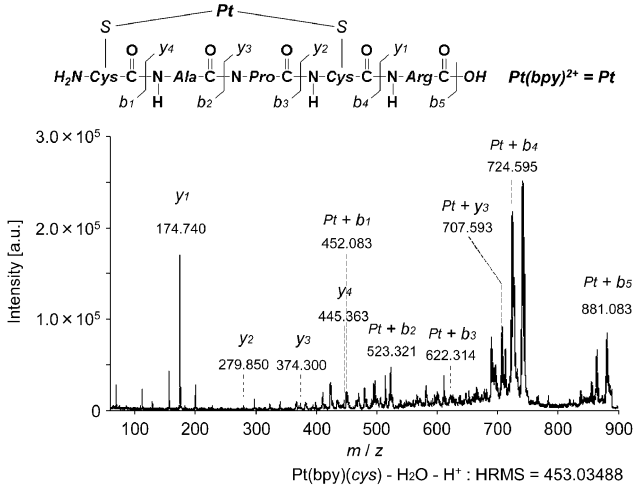

Fig. 6 LIFT-TOF/TOF MS spectrum of **1** digested by chymotrypsin (parent mass: m/z 897.182).

and [Pt(bpy)(Ala-Glu-Trp-*cys*-Ala-Pro-*cys*-Arg) − H_2O + H^+], respectively. In Fig. 6, the peak detected in m/z 724.595 is assigned as the complex between $Pt(bpy)^{2+}$ unit and the N-terminal peptide fragment in which the peptide bond between cysteine and arginine is cleaved. Otherwise, the peaks assigned as the platinum–peptide complex in which the peptide bonds between proline and cysteine, and cysteine and alanine are cleaved, are similarly detectable. Therefore, decomposed fragments containing the $Pt(bpy)^{2+}$ unit having the Cys-Ala-Pro-Cys-Arg sequence were observed to establish the amino acid sequence corresponding to the amino acid sequence of *T. thermophilus* HB8 thioredoxin. Likewise in the LIFT TOF/TOF MS spectra obtained from [Pt(bpy)(Ala-Glu-Trp-*cys*-Ala-Pro-*cys*-Arg) − H_2O + H^+] (Fig. S4, ESI†), the peaks of the platinum complex with peptide fragment in which the peptide bonds are progressively cleaved from C-terminus are detectable. Consequently, the results of UV-visible and MALDI-TOF MS spectra indicate that the $Pt(bpy)^{2+}$ unit binds to the Cys-Ala-Pro-Cys sequence in the active site of thioredoxin. Furthermore, this finding suggests that the bond between platinum and the sulfur of cysteine is not cleaved by chymotryptic digestion or strong acids, such as trifluoroacetic acid, used in MALDI-TOFMS measurements, and is thus very strong.

Catalytic activity of 1

The reduction of glutathione disulfide (GSSG) by thioredoxin has been studied for some thioredoxin systems.[30,31] The reduction rate was determined using wild-type thioredoxin and **1** in the presence of NADPH and *E. coli* thioredoxin reductase. This rate was estimated by measuring the quantity of the consumption for NADPH per minute oxidized in the reaction cycle. Fig. 7 shows the absorbance changes at 340 nm, ascribed to the reduced NADPH. The absorbance at 340 nm in wild-type thioredoxin decreases suddenly, while the decreases of the absorbance in **1** and in the control (without thioredoxin) are very slow. The results yield a rate constant of 3.01 μM min^{-1} in wildtype thioredoxin, 0.33 μM min^{-1} in **1** and 0.34 μM min^{-1} without thioredoxin, respectively. This finding suggests that the enzyme activity of **1** is zero and that the platinum(II) complex acts as an inhibitor of thioredoxin by binding with the active site.

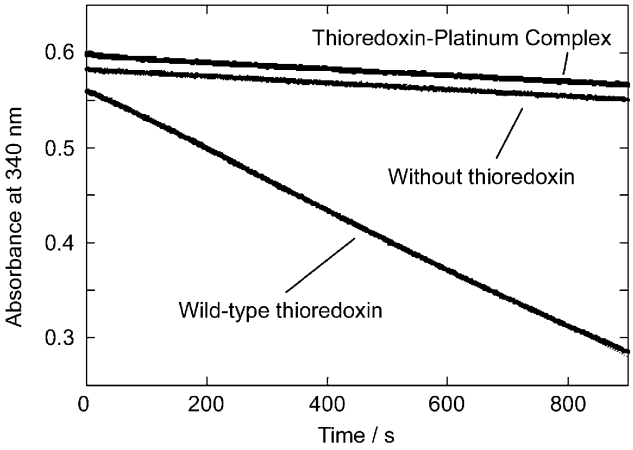

Fig. 7 Estimation of kinetic constants for the reduction of GSSG by *T. thermophilus* HB8 thioredoxin and **1**.

Conclusion

We have demonstrated that the $Pt(bpy)^{2+}$ unit forms a 1 : 1 complex with *Thermus thermophilus* HB8 thioredoxin and binds to the chelating Cys-Ala-Pro-Cys part in the active site as detected by MALDI-TOF MS and UV-visible spectra. We also found that the Pt(bpy)(peptide) fragment is stable during digestion and in strong acid. This method using platinum complex as a labeled compound is applicable for the detection of the active center in proteins, especially for the Cys-involving active center. The enzyme activity of **1** is zero and the platinum(II) complex functions as an inhibitor of thioredoxin. This finding predicts that the resistance to cisplatin is caused by the tight capture of cisplatin with the active site of thioredoxin in cancer cells in which thioredoxin is overexpressed.

Experimental

Preparation and purification of 1

After treatment with dithiothreitol and dialysis, *Thermus thermophilus* HB8 thioredoxin (final concentration: 87.5 pmol μl^{-1}) was mixed with 10 equivalents of $Pt(bpy)(en)^{2+}$ aqueous solution in 0.1 M phosphate buffer (pH 8.0). The mixture solution was incubated at 37 °C for 3 days. Thereafter, the excess platinum complex was removed by ultrafiltration. The mixture of wild-type thioredoxin and **1** was purified by 6% native-polyacrylamide gel electrophoresis (PAGE) using a Bio-rad Model 491 Prep Cell.

Digestion of thioredoxin and 1

To 40 μl of diluted thioredoxin and a solution of **1** (concentration: 40 pmol μl^{-1}) was added urea (6 M) and the mixture was heated at 95 °C for 20 min. Then we added 150 μl of 50 mM NH_4HCO_3 buffer and 1.0 μl of the chymotrypsin solution (containing 2 μg of enzyme) and incubated at 37 °C overnight. After digestion, the peptides were purified with a ZipTip C18 pipette tip.

GHOST (Glutathione as substrate of thioredoxin) assay

In a total assay volume of 3 ml, 10 μl of NADPH (to 100 μM) was mixed with buffer, thioredoxin (to 5.0 μM) and *E. coli* thioredoxin reductase (10 nM). The enzymatic reaction was initiated by adding 150 μl of GSSG (to 1 mM).

Physical measurements

Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) spectra were recorded using a Bruker ultraflex TOF/TOF. MALDI spots were made by mixing 5 μl of 20 pmol μl^{-1} of complex solution or 5 pmol ml^{-1} of digestion solution with 5 μl of matrix solution. 1 μl of this solution was deposited on a stainless steel probe and allowed to air-dry. The matrix solution was 0.07% trifluoroacetic acid and 30% acetonitrile solution saturated α-cyano-4-hydroxycinnamic acid (CHCA) or sinapic acid (SA). UV-visible spectra were recorded on a Shimadzu UV3100PC spectrometer. These measurements were carried out using cells of 0.1- and 1.0-cm pathlength. Circular dichroism (CD) measurements were recorded on a Jasco J-720 w spectropolarimeter. Measurement was carried out using a 0.01 cm pathlength cell at *ca*. 303 K.

Acknowledgements

This work was supported by a Grant-in Aid for Scientific Research on Priority Area (A) (No. 12304040) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. One of the authors (M. K.) expresses his special thanks for the center of excellence (21COE) program "Creation of Integrated EcoChemistry of Osaka University".

References

1 G. Powis and W. R. Montfort, *Annu. Rev. Pharmacol. Toxicol.*, 2001, **41**, 261.
2 S. Kakolyris, A. Giatromanolaki, M. Koukourakis, G. Powis, J. Souglakos, E. Sivridis, V. Georgoulias, K. C. Gatter and A. L. Harris, *Clin. Cancer Res.*, 2001, **7**, 3087.
3 N. B. Arnold, K. Ketterer, J. Kleeff, H. Friess, M. W. Buechler and M. Korc, *Cancer Res.*, 2004, **64**, 3599.
4 H. J. Kim, H. Z. Chae, Y. J. Kim, Y. H. Kim, T. S. Hwang, E. M. Park and Y. M. Park, *Cell Biol. Toxicol.*, 2003, **19**, 285.
5 L.-E. Shao, M. B. Diccianni, T. Tanaka, R. Gribi, A. L. Yu, J. D. Pullen, B. M. Camitta and J. Yu, *Cancer Res.*, 2001, **61**, 7333.
6 Y. Soini, K. Kahlos, U. Napankangas, R. Kaarteenaho-Wiik, M. Saily, P. Koistinen, P. Paakko, A. Holmgren and V. L. Kinnula, *Clin. Cancer Res.*, 2001, **7**, 1750.
7 J. Raffel, A. K. Bhattacharyya, A. Gallegos, H. Cui, J. G. Einspahr, D. S. Alberts and G. Powis, *J. Lab. Clin. Med.*, 2003, **142**, 46.
8 D. T. Lincoln, E. M. Ali Emadi, K. F. Tonissen and F. M. Clarke, *Anticancer Res.*, 2003, **23**, 2425.
9 J. Wang, M. Kobayashi, K. Sakurada, M. Imamura, T. Moriuchi and M. Hosokawa, *Blood*, 1997, **89**, 2480.
10 N. Kawahara, T. Tanaka, A. Yokomizo, H. Nanri, M. Ono, M. Wada, K. Kohno, K. Takenaka, K. Sugimachi and M. Kuwano, *Cancer Res.*, 1996, **56**, 5330.
11 A. Yokomizo, M. Ono, H. Nanri, Y. Makino, T. Ohga, M. Wada, T. Okamoto, J. Yodoi, M. Kuwano and K. Kohno, *Cancer Res.*, 1995, **55**, 4293.
12 T. Sasada, S. Iwata, N. Sato, Y. Kitaoka, K. Hirota, K. Nakamura, A. Nishiyama, Y. Taniguchi, A. Takabayashi and J. Yodoi, *J. Clin. Invest.*, 1996, **97**, 2268.
13 D. L. Kirkpatrick, M. Kuperus, M. Dowdeswell, N. Potier, L. J. Donald, M. Kunkel, M. Berggren, M. Angulo and G. Powis, *Biochem. Pharmacol.*, 1998, **55**, 987.
14 P. Wipf, S. M. Lynch, A. Birmingham, G. Tamayo, A. Jimenez, N. Campos and G. Powis, *Org. Biomol. Chem.*, 2004, **2**, 1651.
15 M. Pallis, T. D. Bradshaw, A. D. Westwell, M. Grundy, M. F. G. Stevens and N. Russell, *Biochem. Pharmacol.*, 2003, **66**, 1695.
16 S. Gromer, S. Urig and K. Becker, *Med. Res. Rev.*, 2003, **24**, 40.
17 F. Rollin-Genetet, C. Berthomieu, A.-H. Davin and E. Quemeneur, *Eur. J. Biochem.*, 2004, **271**, 1299.
18 S. Gromer, L. D. Arscott, C. H. Williams, Jr., R. H. Schirmer and K. Becker, *J. Biol. Chem.*, 1998, **273**, 20096.
19 K. Becker, C. Herold-Mende, J. J. Park, G. Lowe and R. H. Schirmer, *J. Med. Chem.*, 2001, **44**, 2784.
20 S. Bonse, J. M. Richards, S. A. Ross, G. Lowe and R. L. Krauth-Siegel, *J. Med. Chem.*, 2000, **43**, 4812.
21 J. A. Weinstein, N. N. Zheligovskaya, M. Y. Mel'nikov and F. Hartl, *J. Chem. Soc., Dalton Trans.*, 1998, 2459.
22 J. A. Weinstein, A. J. Blake, E. S. Davies, A. L. Davis, M. W. George, D. C. Grills, I. V. Lileev, A. M. Maksimov, P. Matousek, M. Y. Mel'nikov, A. W. Parker, V. E. Platonov, M. Towrie, C. Wilson and N. N. Zheligovskaya, *Inorg. Chem.*, 2003, **42**, 7077.
23 S. K. Katti, D. M. LeMaster and H. Eklund, *J. Mol. Biol.*, 1990, **212**, 167.
24 A. Weichsel, J. R. Gasdaska, G. Powis and W. R. Montfort, *Structure*, 1996, **4**, 735.
25 R. Friemann, H. Schmidt, S. Ramaswamy, M. Forstner, R. L. Krauth-Siegel and H. Eklund, *FEBS Lett.*, 2003, **554**, 301.
26 H. J. Dyson, G. P. Gippert, D. A. Case, A. Holmgren and P. E. Wright, *Biochemistry*, 1990, **29**, 4129.
27 J. D. Forman-Kay, G. M. Clore, P. T. Wingfield and A. M. Gronenborn, *Biochemistry*, 1991, **30**, 2685.
28 M.-F. Jeng, A. P. Campbell, T. Begley, A. Holmgren, D. A. Case, P. E. Wright and H. J. Dyson, *Structure*, 1994, **2**, 853.
29 J. Qin, G. M. Clore and A. M. Gronenborn, *Structure*, 1994, **2**, 503.
30 S. Gromer, H. Merkle, R. H. Schirmer and K. Becker, *Methods Enzymol.*, 2002, **347**, 382.
31 S. M. Kanzok, S. Rahlfs, K. Becker and R. H. Schirmer, *Methods Enzymol.*, 2002, **347**, 370.