Human Thioredoxin Reductase Is Efficiently Inhibited by (2,2′:6′,2″-Terpyridine)platinum(II) Complexes. Possible Implications for a Novel Antitumor Strategy

Katja Becker,*,† Christel Herold-Mende,‡ Jenny J. Park,§ Gordon Lowe,§ and R. Heiner Schirmer||

Interdisciplinary Research Center, Giessen University, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany, Department of Neurosurgery and Department of Head and Neck Surgery, Heidelberg University, Im Neuenheimer Feld 400, D-69120 Heidelberg, Germany, Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford, OX1 3QY, U.K., and Center of Biochemistry, Heidelberg University, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany

Received June 23, 2000

Malignant neoplasms of the brain represent the second leading cause of cancer-related mortality in children under the age of 15. The prognosis of patients with glioblastoma multiforme, the most malignant type of gliomas, remains poor offering a median survival time of only 1 year. (2,2′:6′,2″-Terpyridine)platinum(II) complexes are known to possess DNA-intercalating activity and have been shown to be potential chemotherapeutic agents. In the present study we identified the selenoenzyme thioredoxin reductase (TrxR) as a major target of (2,2′:6′,2″-terpyridine)platinum(II) complexes. New complexes were synthesized in order to optimize this inhibition. The NADPH-reduced enzyme is inhibited almost stoichiometrically by the complexes involving a reversible competitive and an irreversible tight-binding component. For the most potent inhibitor, *N*,*S*-bis(2,2′:6′,2″-terpyridine)platinum(II)-thioacetimine trinitrate, the K_i for the competitive component of the inhibition is 4 nM and the IC_{50} for the tight-binding component is 2 nM after an incubation time of 5 min. The closely related but non-selenium-containing enzyme glutathione reductase is much less inhibited (by a factor of >1000). The platinum complexes were found to strongly inhibit the proliferation of three different glioblastoma cell lines as well as of two different head-and-neck squamous carcinoma cell lines. In a glioblastoma cell culture, less than 10 μM of a platinum(II) compound caused an initial drop of hTrxR activity which was followed by an increase of activity in the surviving cells. A 10 μM inhibitor added every 24 h led to 4% residual hTrxR activity but 100% glutathione reductase activity in the cells surviving for 67 h. The potential of (2,2′:6′,2″-terpyridine)platinum(II) complexes acting simultaneously at two different intracellular targets—hTrxR and DNA—as antitumor agents is discussed.

Introduction

Human thioredoxin reductase (TrxR; NADPH + H^+ + Trx-S_2 ⇌ $NADP^+$ + Trx-$(SH)_2$) is an FAD-dependent homodimeric oxidoreductase closely related to glutathione reductases and lipoamide dehydrogenases.[1] A special feature of mammalian TrxRs is, however, a C-terminal -Gly-*Cys*-*Sec*-Gly-COOH sequence. This additional redox center, and particularly the selenocysteine, is essential for the catalytic mechanism of the enzyme (cf. refs 2 and 3). It is located on the flexible C-terminal extension of the protein, picks up electrons from the active site, and according to the current hypothesis, transfers them to various substrates.[4−7] Apart from the 12 kDa protein thioredoxin, hTrxR is capable of reducing a whole range of other substrates including low M_r compounds like lipoamide, hydrogen peroxide, *S*-nitrosoglutathione, but also NK-lysin, protein disulfide isomerase, and plasma glutathione peroxidase.[5,6,8] Reduced thioredoxin (Trx) provides reducing equivalents for a number of processes such as the formation of deoxyribonucleotides by ribonucleotide reductase, one of the key steps in DNA synthesis and therefore also in cell proliferation. Furthermore, the thioredoxin system regulates the activity of transcription factors, modulates protein biosynthesis and export protein structures, regulates the activity of other enzymes, and represents one of the major intracellular antioxidant cornerstones.[5−9] Many tumor cells are known to have severalfold-increased Trx and TrxR levels.[8,10,11] In addition, some tumor cells can secrete reduced Trx which then acts as an autocrine growth stimulator.[8,12−16] It is therefore not surprising that the cytostatic agent carmustin (BCNU) and also the gold-containing antirheumatic and antineoplastic compound auranofin have been shown to efficiently inhibit hTrxR.[17−19]

Malignant neoplasms of the brain are the second leading cause of cancer-related mortality in children under the age of 15 and the third and fourth leading cause in men and women of the age group between 15 and 34.[20,21] Among these neoplasms malignant gliomas are the most common primary brain tumors, accounting for 60% of intracranial neoplasms in adults.[22−25] To date, the prognosis of patients with glioblastoma mul-

* To whom correspondence should be addressed. Phone: +49-(0)-641-9939120. Fax: +49-(0)641-9939129. E-mail: becker.katja@gmx.de.
† Giessen University.
‡ Department of Neurosurgery and Department of Head and Neck Surgery, Heidelberg University.
§ University of Oxford.
|| Center of Biochemistry, Heidelberg University.

10.1021/jm001014i CCC: $20.00
Published on Web 07/20/2001

tiforme (GBM), the most malignant type of gliomas remains poor, offering a median survival time of only 1 year, despite multimodality therapy, including surgical resection as well as radio- and chemotherapy.[21,26−28] The best results of adjuvant chemotherapy are achieved with a nitrosourea chemotherapy, either BCNU or a combination of procarbazine, lomustine (CCNU), and vincristine, known as PCV-3-therapy.[29] But so far, mainly patients with anaplastic oligodendrogliomas benefit from nitrosourea therapy while the effects in patients with GBM are only marginal.[30]

Platinum-containing compounds, like the established cytostatic agent cisplatin, are well-known antitumor agents. However, of the thousands of platinum compounds evaluated as antitumor agents, only a very small fraction has shown sufficient promise during preclinical evaluation.[31] Already 25 years ago, (2,2′:6′,2″-terpyridine)platinum(II) complexes were shown to bind to double-stranded DNA by intercalation.[32] Since these first reports, a range of derivatives of the complexes have been synthesized and the mechanism of interaction between the compounds and DNA has been studied.[33,34] Very recently, (2,2′:6′,2″-terpyridine)platinum(II) complexes were demonstrated to be cytotoxic to *Trypanosoma* and *Leishmania* parasites[35] as well as to human ovarian carcinoma.[36] Ligand substitution reactions, as displayed by the platinum complexes, are not limited to DNA, and similar processes can be expected with nucleophilic functional groups, which are vital for enzyme activity, such as selenols, thiols, and imidazoles.[37]

In the present study, we identified the human selenoenzyme thioredoxin reductase as a major target for the action of (2,2′:6′,2″-terpyridine)platinum(II) complexes. Furthermore, the proliferation of different glioblastoma cell lines was shown to be effectively inhibited by these compounds.

Results

Inhibition of Human Thioredoxin Reductase by Platinum Complexes. The inhibition of hTrxR by the (2,2′:6′,2″-terpyridine)platinum(II) complexes (Figure 1) shows an inactivation pattern characterized by a reversible component competing with the substrate DTNB as well as a tight-binding component. For determining the inhibition constants (K_i) for the reversible component of inhibition, approximately 2 nM hTrxR was mixed with variable concentrations of the disulfide substrate DTNB (50 μM to 1 mM) and one inhibitor concentration (10 nM to 1 μM depending on the inhibitor) in assay buffer. The reactions were started with 200 μM NADPH at 25 °C. In parallel the K_m value for DTNB in the absence of inhibitor was determined. For one inhibitor, compound 1, K_m values were determined at different inhibitor concentrations. When representing the data in a Cornish−Bowden diagram, which plots inhibitor concentrations against the ratio substrate concentration/enzyme activity, the competitive component of the inhibition becomes evident at DTNB concentrations of ≤400 μM, as indicated by parallel lines (Figure 2). Therefore, K_i values for competitive inhibition by the different platinum complexes were calculated according to the equation $K_i = K_m[I]/(K_m' - K_m)$. The data are summarized in Table 1.

Table 1. Inhibition of Human Thioredoxin Reductase (hTrxR) by Platinum Complexes[a]

compd	K_m' of DTNB [μM]	[I] for K_m' determination [μM]	K_i, competitive [nM]	IC_{50} in preincubation assay [nM]	hGR inhibition by 10 μM Pt(II) compd [%]
1	394	0.1	35	5	13
2	399	1	348	35	7
3	4738	1	22	30	55
4	177	0.1	139	200	49
5	1007	0.1	11	3	20
6	1055	0.05	5	4	90
7	715	0.1	17	7	66
8	247	0.01	7	6	47
9	2723	0.1	4	2	30
10	408	1	337	25	35

[a] The K_m value for DTNB in the absence of inhibitor was 103 ± 20 μM. Given are the K_m' value for DTNB in the presence of the inhibitor, the inhibitor concentration for which the K_m' was determined, the calculated K_i value when assuming competitive inhibition, the IC_{50} value of the platinum complexes for inhibiting hTrxR. The IC_{50} values were obtained by 2 min preincubation of the enzyme with NADPH and varying inhibitor concentrations under standard assay conditions prior to the addition of DTNB. The last column demonstrates the relatively weak inhibition of human glutathione reductase (hGR) by 10 μM platinum complexes after 2 min preincubation in the enzyme assay mixture.

To characterize the tight-binding component of the inhibition, 2 nM hTrxR in assay buffer was reduced with 200 μM NADPH; then the respective inhibitor was added and the mixture was incubated for 5 min at 25 °C. Subsequently hTrxR activity was measured by adding 3 mM DTNB. IC_{50} values were calculated from the dose−response curves (Table 1). The tight-binding inhibition of hTrxR is a fairly rapid process. As determined for compound **1**, the $t_{1/2}$ for enzyme inhibition is 25 s when 2 nM hTrxR and 10 nM inhibitor are allowed to react; this corresponds to a second-order rate constant of approximately 2×10^6 M^{-1} s^{-1} at 293 K. It should be noted that reduction of the enzyme, in our case by 200 μM NADPH, is a prerequisite for tight-binding inhibition. When hTrxR was preincubated with the platinum complexes in the absence of NADPH, less than 10% inhibition was observed.

Reversibility of the Inhibition. The reversibility of the inhibition of hTrxR by the platinum complexes was tested in detail for compound **8**. The enzyme was incubated with 200 nM inhibitor in the presence of 100 μM NADPH representing the physiologic intracellular concentration. After 5 min 83% of the enzyme was inhibited. In a parallel incubation that additionally contained 2 mM reduced glutathione, 66% enzyme inhibition was determined. Aliquots of the first sample were then incubated for 20 min at 25 °C with various thiol compounds. A 2 mM GSH sample resulted in no reactivation; <20% and <10% activity were observed with 1 mM 2,3-dithiopropanol (British Antilewisite, BAL) and 1 mM DTT, respectively. Furthermore, dialysis of the sample against TrxR assay buffer did not lead to more than 15% recovery of activity. In conclusion, the inhibition of hTrxR by the (2,2′:6′,2″-terpyridine)platinum(II) complexes is very stable and unlikely to be reversible under in vivo conditions within a biologically meaningful time.

Inhibition of Human Glutathione Reductase by Platinum Complexes. To test the specificity of hTrxR inhibition by the platinum complexes, we also determined the inhibitory effects of the compounds on the

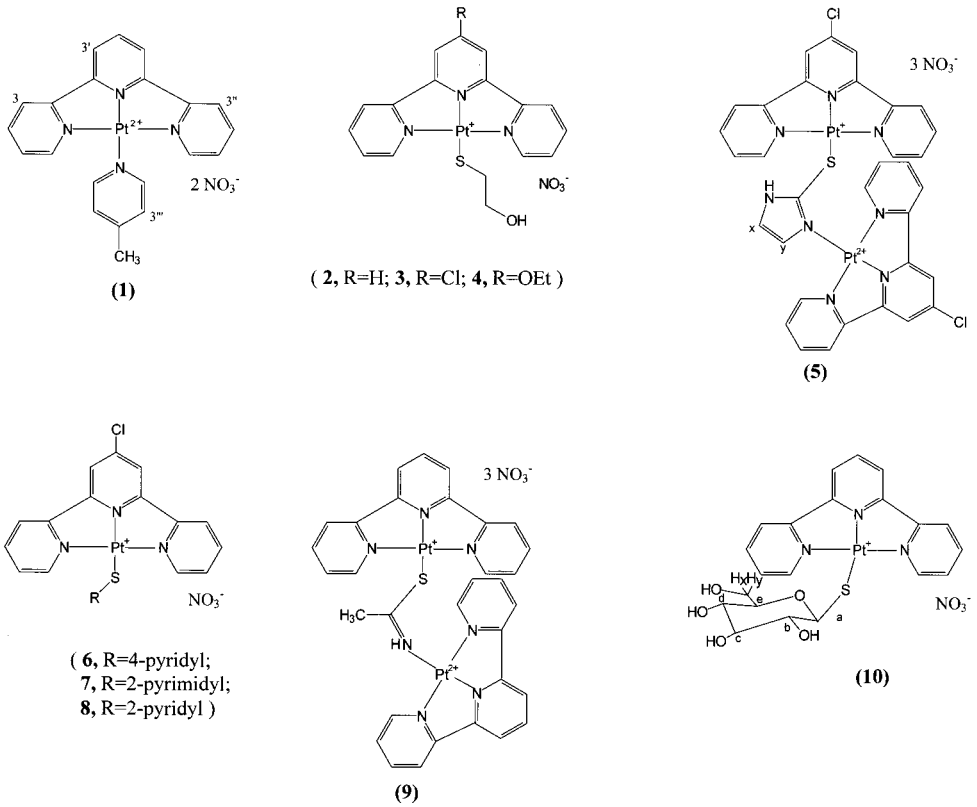

Figure 1. Structure of the ten (2,2′:6′,2″-terpyridine)platinum(II) complexes tested. The numbers given to the compounds in the figure are also used in the text.

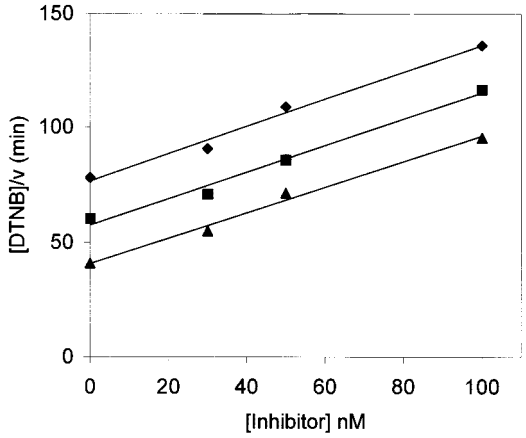

Figure 2. Cornish−Bowden diagram for the inhibition of human thioredoxin reductase by platinum complex **1**. At concentrations of the substrate DTNB in the K_m range, the reversible competitive component of inhibition becomes evident. Upper line is for 400 μM DTNB, middle line for 300 μM DTNB, and lower line for 200 μM DTNB.

closely related human enzyme glutathione reductase. The inhibitor was studied directly in the assay on the NADPH-reduced enzyme after a preincubation time of 5 min. As shown in the last column of Table 1, hGR could be inhibited by the platinum complexes; however, the inhibition was by at least three orders of magnitude weaker than for TrxR.

Inhibition of Tumor Cell Proliferation by Platinum Complexes. We tested the effects of compounds **6** and **9**, which were the two most effective hTrxR inhibitors among the platinum complexes studied (Table 1), on the proliferation of three different highly malignant glioblastoma cell lines and two different head-and-neck squamous carcinoma cell lines. Single-dose application was studied as well as the application of three identical doses applied at 24 h intervals. As shown in Table 2, a single dose of compound **6** or compound **9** inhibited the growth of all five tumor cell lines with IC_{50} values ranging between 2.5 and 10.5 μM. Three subsequent applications (3 × 10 μM) of the compounds resulted in >95% growth inhibition in 9 out of 10 experiments (data not shown). The two inhibitors had similar effects and acted dose-dependently on all tumor cell lines.

Table 2. Antiproliferative Effects of Compounds **6** and **9** on Different Malignant Cell Lines[a]

	tumor cell line				
	NCH37	NCH82	NCH89	HNO97	HNO199
IC_{50} for **6** [μM]	10.5 ± 3.1	7.4 ± 2.2	2.5 ± 1.3	5.5 ± 1.8	9.2 ± 2.6
IC_{50} for **9** [μM]	5.7 ± 1.8	3.9 ± 1.3	2.5 ± 1.2	4.8 ± 1.3	6.2 ± 2.1

[a] Shown is the growth inhibition on three glioma cell lines (NCH37, NCH82, and NCH89) and on two head-and-neck squamous carcinoma cell lines (HNO97 and HNO199). Given are IC_{50} values ± SD of the compounds, which is the drug concentration leading to 50% growth inhibition relative to the proliferation of inhibitor-free control cultures.

TrxR and GR Activity in Cell Cultures after Incubation with Compound 6. As shown in Table 3 for compound **6**, the (terpyridine)platinum(II) complex has an immediate dose-dependent effect on the TrxR activity of the glioblastoma cell line NCH37. A single application of 1 μM reduced TrxR activity to 50% within 30 min and 5 μM reduced the activity to 30%. In the surviving cells (enzyme activity was only determined in intact cells!), TrxR activity increased again over time as indicated by a value of 90% of controls after 270 min and of 160% after 67 h. When the cells with a single

Table 3. TrxR and GR Activities after Exposure of NCH37 Cells to Compound **6**[a]

treatment with compound **6**	TrxR activity [%]	GR activity [%]
controls	100	100
1 μM (5 μM) for 30 min	50 (30)	90
1 μM for 90 min	50	90
1 μM for 270 min	90	110
1 μM for 67 h	160	220
5 μM for 67 h	130	200
10 μM for 67 h	80	260
3 × 1 μM (for 67 h)[b]	110	180
3 × 5 μM (for 67 h)[b]	60	220
3 × 10 μM (for 67 h)[b]	5	180

[a] All values are given as a percentage of untreated controls. Enzyme activities were determined in mU/10^6 cells; 100% refers to 3.9 mU in the case of TrxR and to 3.4 mU in the case of GR. Since dying cells release their cytosolic enzymes, enzyme activities refer only to intact cells that have survived treatment and not to damages cells. This most likely explains the long-term induction of both enzyme activities. The time points refer to samples that were taken 30, 90, and 270 min and 67 h after (first) addition of the platinum complex. Given are mean doses of three independent experiments. [b] The doses given at 0, 24, and 48 h accumulate.

dose or with three subsequent accumulating doses of compound **6** were treated, a dose-dependent reduction of enzyme activity was determined after 67 h in both cases. Glutathione reductase activity, which was stable over the first hours of the experiment, was found to be induced by the treatment. This induction, by a factor of approximately 2 after 67 h, did not depend on the inhibitor concentration.

Discussion

The Thioredoxin System as a Chemotherapeutic Drug Target. The role of the thioredoxin system in tumor research is Janus faced. Oxidative stress is considered a major trigger for DNA damage and carcinogenesis. Therefore, the antioxidant Trx system can be regarded as a tumor-preventive system. However, once a tumor has been established, the malignant cells require a constant deoxyribonucleotide supply that in turn depends on a highly active Trx/TrxR system.[8] Furthermore, the thioredoxin system provides protection from NK-lysin, tumor necrosis factor-α, and reactive oxygen species.[38−40] It is therefore not surprising that tumor cells have been reported to express severalfold-increased TrxR levels and that some potent antineoplastic agents such as carmustine, fotemustine,[18,19,41,42] and cisplatin[43] are effective inhibitors of mammalian TrxRs. Furthermore, cellular thioredoxin levels have been shown to be correlated with drug resistance to cisplatin, mitomycin C, doxorubicin, and etoposide.[43,44] It should also be noted that many transcription factors such as NF-κB, NF-Y, and p53 are redox-dependent in their activity.[45,46] In the case of NF-κB and p53, links to the Trx system have already been established.[47,48]

Human Trx was first purified from plasma of patients and referred to as adult T-cell leukemia-derived factor (ADF).[15] Trx levels in the plasma of patients suffering from adult T-cell leukemias correlate with chemoresistance of the tumor cells to adriamycin. As mentioned above, cell-secreted Trx in its reduced state can also act as an autocrine growth factor, especially for tumor cells.[14] These findings again render the Trx system a prime antineoplastic drug target. Furthermore, as published in a case report,[49] autoantibodies to TrxR were found in a patient with ovarian cancer, suggesting their potential value as tumor markers. Apart from malignancies, TrxR inhibition is likely to be beneficial in the treatment of chronic inflammatory diseases like rheumatoid arthritis and Sjögren's syndrome. The antirheumatic gold compounds auranofin and aurothioglucose have been characterized as specific and highly effective tight-binding inhibitors of hTrxR, the formal K_i being approximately 4 nM.[19] The proposed mechanism of action is the reaction with the C-terminally-located selenocysteine of hTrxR, which is essentially involved in catalysis.[4−6] That this inhibition is also effective in vivo has recently been demonstrated in the mouse model.[50]

hTrxR Inhibition by Platinum Complexes. As summarized in Table 1, hTrxR is inhibited by (2,2′:6′,2″-terpyridine)platinum(II) complexes in a dose-dependent manner. The type of inhibition can be divided into a reversible competitive component and a practically irreversible tight-binding component. Since both types of inhibition affect the enzyme in parallel, a specific experimental setup was chosen (see Results) to differentiate as much as possible between them. K_m values for the artificial substrate DTNB markedly increased in the presence of the inhibitors, and as demonstrated in Figure 2, at DTNB concentrations of ≤400 μM the competitive inhibition becomes clearly evident. We therefore determined for each platinum complex the K_m' value in the presence of an appropriate inhibitor concentration and calculated the K_i for competitive inhibition. All K_i values determined are in the nanomolar range, pointing to pronounced enzyme inhibition. In comparison with previously published data, the K_m for DTNB in the absence of inhibitor was very low (103 μM instead of 400 μM[19]). This value was repetitively determined for the particular enzyme fraction used. We explain this observation by the fact that the enzyme was freshly prepared and of high purity not only according to a silver-stained SDS gel but also according to spectral characteristics.[4,19] With all inhibitors IC_{50} values for the irreversible inhibition were calculated from dose−response curves (Table 1). This inactivation takes place within seconds to minutes; as determined for compound **1**, the $t_{1/2}$ for enzyme inhibition is 25 s in the presence of 10 nM inhibitor. The IC_{50} values determined were all in the nanomolar range; for six out of the ten platinum complexes, the values were lower than 10 nM. These data demonstrate that the inhibition takes place in almost stoichiometric quantities. In contrast, for inhibition of human glutathione reductase, a mechanistically related enzyme, >1000-fold higher inhibitor concentrations were required; this difference points to a highly specific hTrxR inhibition by the platinum complexes.

Human glutathione reductase and hTrxR are FAD-dependent homodimers with a similar architecture.[4] Both enzymes can be inactivated by BCNU, which, as demonstrated for hGR and postulated for hTrxR, carbamoylates a catalytic site thiol.[17,18,51] The major difference between the two oxidoreductases is the C-terminally-located Cys−Sec pair of hTrxR, which acts as an additional redox center and which is absent in glutathione reductase.[3,4] According to the present hypothesis, this redox center is positioned at a flexible

extension of the protein that is able to pick up electrons from the catalytic site dithiol and to transfer them to thioredoxin disulfide or one of the other numerous substrates.[2−6] As in the case of auranofin, the most probable target of (2,2′:6′,2″-terpyridine)platinum(II) complexes is the penultimate selenocysteine.[19] This hypothesis is supported by the facts that only NADPH-reduced hTrxR is inactivated—which indicates that a redox-active cysteine or selenocysteine is involved in the reaction—and that the inhibition of hGR by the platinum(II) complexes is by orders of magnitude weaker than for hTrxR.

(2,2′:6′,2″-Terpyridine)platinum(II) Complexes as Potential Cytostatic Agents. The interest in platinum-based antitumor drugs has its origin in the 1960s with Rosenberg's discovery of the inhibition of cell division by platinum complexes. The ensuing systematic experiments led to the development of *cis*-diamminedichloroplatinum(II) (cisplatin), which today is one of the three most widely administered antitumor drugs in the world.[31] However, platinum compounds that are presently in clinical use, like cisplatin and carboplatin, have severe side effects and their applicability is still limited to a relatively narrow range of tumors. Therefore, the search continues for a less toxic, orally active compound that is not cross-resistant with cisplatin and carboplatin.[31]

(2,2′:6′,2″-Terpyridine)platinum(II) complexes were shown to intercalate with double-stranded DNA.[32,33] Very recently, (2,2′:6′,2″-terpyridine)platinum(II) complexes have been demonstrated to be cytotoxic to human ovarian carcinoma and to have little or no cross-resistance with cisplatin or doxorubicin.[36] Furthermore, the complexes exhibit antiprotozoal activity against *Trypanosoma* and *Leishmania* parasites.[35] Ligand substitution reactions of platinum complexes are not limited to DNA but can be expected to occur with nucleophilic functional groups of proteins like thiols or selenols.[35,37] Potential positive effects of the platinum−sulfur interaction, such as the possible prevention of side effects and optimal transport of the compounds, have been discussed recently.[37] The high affinity of platinum complexes to thiols and selenols has also been underlined by the fact that cisplatin is an inhibitor of mammalian TrxRs.[43] Furthermore, high cellular thioredoxin levels are associated with drug resistance to cisplatin, mitomycin C, doxorubicin, and etoposide.[44]

As shown in Table 1, the (2,2′:6′,2″-terpyridine)-platinum(II) complexes studied here strongly inhibit the human selenoenzyme TrxR both competitively and irreversibly. The inhibitory effects on the enzyme have been optimized by synthesis of different derivatives. This approach resulted in a group of highly effective hTrxR inhibitors that inactivate the enzyme irreversibly in practically stoichiometric quantities and within seconds. The disulfide reductases glutathione reductase and thioredoxin reductase are known to be inhibited by the cytostatic agent BCNU, which is beneficial for the treatment of glioblastoma.[17,29] We therefore tested the effects of the two platinum(II) complexes (compounds **6** and **9**) with the highest TrxR inhibition capacity on the growth of three different highly malignant glioblastoma cell lines. In parallel, two head-and-neck squamous carcinoma cell lines were studied. As shown in Table 2, within 67 h tumor cell proliferation was suppressed by 50% even by a single dose of either compound ($\leq 10\ \mu$M). When applying three subsequent doses of 10 μM inhibitor in 24 h intervals, tumor cell proliferation was suppressed by ≥95% in 9 out of 10 experiments. As demonstrated in Table 3 for compound **6**, the platinum complex had an immediate and dose-dependent effect on the TrxR activity of the glioblastoma cell line NCH37. Also after 67 h, a dose-dependent reduction of TrxR activity was observed. At lower doses this inhibitory effect is likely to be antagonized by an induction of enzyme activity in the cells that survived treatment. Glutathione reductase activity was unaltered over the first hours of the experiment but was, however, increased after 67 h.

Conclusion

To simultaneously decrease DNA synthesis, antioxidant defense and potential autocrine growth stimulation of a tumor cell by the inactivation of *one* single enzyme, namely, hTrxR, is a tempting approach. Since (2,2′:6′,2″-terpyridine)platinum(II) complexes are furthermore known to bind to DNA, a second, completely different target is attacked at the same time. This results in the potentiation of the cytostatic activity and is likely to prevent the development of drug resistance. Since platinum complexes inactivate hTrxR in almost stoichiometric quantities and the inactivation seems to be irreversible under in vivo conditions, it will be difficult to generate more potent enzyme inhibitors. We consider that the very high specificity of the (2,2′:6′,2″-terpyridine)platinum(II) thiolate complexes for hTrxR (compared to hGR) is due to the high affinity of thiols for platinum(II) in the complexes shown in Figure 1 (except for compound **1**). This suggests that it is the selenocysteine residue that is the site of platination in hTrxR and that the mechanism involves a selenolate−thiolate ligand exchange of the platinum(II) compounds. Further studies will be carried out on bioavailability, pharmacokinetics, toxicity, and in vivo activity of these complexes.

To facilitate efficient and cost-effective inhibitor screening also in high throughput systems, alternative substrates of hTrxR have been developed.[52] The structure of these compounds, which have favorable K_m values, is based on dithiobisnitrobenzoate (DTNB), and a respective microtiter plate assay system for drug screening has been developed. Since mouse TrxR has been characterized as a BCNU-sensitive selenoprotein[18] that is likely to be inhibited by platinum complexes, the mouse model will contribute reliable data on bioavailability, pharmacokinetics, and toxicity when testing platinum complexes.

Experimental Procedures

Materials. Human thioredoxin reductase (hTrxR) was purified from placenta as described by Gromer et al. in 1998.[19] Human glutathione reductase (hGR) was produced and isolated according to Nordhoff et al. in 1993.[53] Recombinant *E. coli* thioredoxin (EcTrx) with an ϵ_{280nm} of 13.6 $mM^{-1}\,cm^{-1}$ was kindly provided by Prof. Charles Williams, Ann Arbor, MI. The other substrates for the enzymatic assays were purchased from Boehringer, Serva, and Sigma. All reagents were of the highest purity available.

Enzyme Assays. All assays were conducted at 25 °C in a total assay volume of 1 mL. For determining TrxR activity the

DTNB reduction assay was employed.[19,54] The enzyme was added to an assay mixture consisting of 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, and 3 mM DTNB (100 mM stock solution in DMSO). After the reaction was initiated with the addition of NADPH (200 μM final concentration), the increase in absorbance at 412 nm was monitored. One enzyme unit is defined as the NADPH-dependent production of 2 μmol 2-nitro-5-thiobenzoate (ϵ_{412nm} of 13.6 mM^{-1} cm^{-1}) per min. For the determination of K_m values, the assay mixtures contained varying concentrations of the respective substrates.

The glutathione reductase assay mixture consisted of 47 mM potassium phosphate, 1 mM EDTA, 200 mM KCl, pH 6.9, and 100 μM NADPH. After addition of hGR, the assay was started with 1 mM GSSG, and the consumption of NADPH was monitored as the decrease in absorbance at 340 nm.[53] Protein concentrations were determined on the basis of their specific absorbance at 280 nm and (for the flavoenzymes) at 463 nm.[4,53]

NMR Spectra and Mass Spectrometry. ^{1}H NMR spectra were recorded on a Bruker AM 400 MHz spectrometer at 300 K by Mrs. Elizabeth McGuiness. Samples were run in deuterium oxide (D_2O) and referenced to dioxane (3.75 ppm). Chemical shifts are expressed in ppm. Abbreviations for multiplicity are the following: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. ^{1}H NMR spectral data are expressed in the order of chemical shift, multiplicity, coupling constant, relative intensity, and assignment. Electrospray mass spectrometry was carried out by Dr. Robin T. Aplin on a VG Biotech Bio-Q spectrometer using a dilute solution of the sample in methanol/water.

Synthesis of (2,2′:6′,2″-Terpyridine)platinum(II) Complexes. The ten (2,2′:6′,2″-terpyridine)platinum(II) complexes tested as inhibitors of human thioredoxin reductase and as antitumor agents are numbered **1**−**10**.

4-Picoline-(2,2′:6′,2″-terpyridine)platinum(II) Dinitrate (1). This was prepared by a modification of the method of Lowe and Vilaivan[55] using silver nitrate in place of silver tetrafluoroborate. It was a beige solid (76.3% yield). ^{1}H NMR (500 MHz, D_2O): δ_H 2.64 (3H, s, CH_3), 7.74 (4H, m, H5,5″, H3‴,5‴), 7.84 (2H, d, J = 5.5 Hz, H6,6″), 8.39 (2H, d, J = 7.5 Hz, H3,3″), 8.43 (2H,d, J = 7.5 Hz, H4,4″), 8.55 (2H, s, H3′,5′), 8.92 ppm (2H, d, J = 6.5 Hz, H2‴, 6‴). ESMS: m/z 278.4 (M^{2+}, 100%).

The syntheses of complexes **2**−**4** were reported elsewhere.[56]

2-Mercaptoimidazole Bis[(4′-chloro-2,2′:6′,2″-terpyridine)platinum(II)] Trinitrate (5). A solution of silver nitrate (64.6 mg, 0.38 mmol) in acetone/water (4:1, 0.6 mL) was added dropwise to a suspension of diiodo-1,5-cyclooctadieneplatinum(II) (99.6 mg, 0.18 mmol) in acetone/water (4:1, 0.6 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The silver iodide precipitate was discarded. The supernatant was added to a suspension of 4′-chloro-2,2′:6′,2″-terpyridine (38.6 mg, 0.144 mmol) in acetonitrile (0.3 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The supernatant was removed and discarded. The pellet was washed with acetonitrile/ether (1:3, 2 × 1.5 mL) and then dissolved in water (0.75 mL). A solution of 2-mercaptoimidazole (6.01 mg, 0.060 mmol) in water (3 mL) was added. The mixture was vortexed and then sonicated for 1.5 h. The mixture was added dropwise to ether/acetone (1:1, 20 mL) to precipitate the complex. The solid was washed with ether/acetone (1:1, 4 × 20 mL) and then dried to yield 2-mercaptoimidazole bis[(4′-chloro-2,2′:6′,2″-terpyridine)platinum(II)] trinitrate (66 mg, 96%) as a dark-purple solid. The product was purified by dissolving the solid in hot methanol/water (1:1) and reprecipitating from ether/acetone (1:1, 25 mL). ^{1}H NMR (400 MHz, D_2O): δ 8.78 (2H, d, J = 5.6 Hz, H6, H6″), 8.47 (2H s, H3′, H5′), 8.46 (2H, s, H3′, H5′), 8.34−8.27 (4H, m, 2 × H4, 2 × H4″), 8.08 (2H, d, J = 8.3 Hz, H3, H3″), 8.05 (2H, d, J = 8.2 Hz, H3, H3″), 7.94 (2H, d, J = 5.6 Hz, H6, H6″), 7.60−7.54 (4H, m, 2 × H5, 2 × H5″), 7.51 (1H, apparent broad s, either H_x or H_y), 7.34 (1H, apparent broad s, either H_x or H_y). ESMS (1:1 MeOH/H_2O, CV = 25 V): m/z 341.6 (MH^{3+}, 100%), 511.6 (M^{2+}, 15%).

4-Mercaptopyridine (4′-Chloro-2,2′:6′,2″-terpyridine)platinum(II) Nitrate (6). A solution of silver nitrate (37.0 mg, 0.218 mmol) in acetone/water (4:1, 0.5 mL) was added dropwise to a suspension of diiodo-1,5-cyclooctadieneplatinum-(II) (55.3 mg, 0.100 mmol) in acetone/water (4:1, 0.5 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The silver iodide precipitate was discarded. The supernatant was added to a suspension of 4′-chloro-2,2′:6′,2″-terpyridine (21.4 mg, 0.080 mmol) in acetonitrile (0.25 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The supernatant was removed and discarded. The pellet was washed with acetonitrile/ether (1:3, 2 × 1.5 mL) and then dissolved in water (0.75 mL). A solution of 4-mercaptopyridine (13.3 mg, 0.12 mmol) in methanol/water (1:1, 1.0 mL) was added. The mixture was vortexed and then sonicated for 1.5 h. The mixture was added dropwise to ether/acetone (1:1, 20 mL) to precipitate the complex. The solid was washed with ether/acetone (1:1, 4 × 20 mL) and then dried to yield 4-mercaptopyridine (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) nitrate (45 mg, 89%) as an orange-brown solid. The product was purified by dissolving the solid in hot methanol/water (1:1) and reprecipitating from ether/acetone (1:1, 25 mL). ^{1}H NMR (400 MHz, D_2O): δ 8.83 (2H, d, J = 5.5 Hz, H6, H6″), 8.55 (2H, s, H3′, H5′), 8.39 (2H, apparent t, J = 7.9 Hz, H4, H4″), 8.28 (2H, d, J = 7.7 Hz, H3, H3″), 8.00 (4H, AA′BB′m, H2‴, H3‴, H5‴, H6‴), 7.71 (2H, m, H5, H5″). ESMS (1:1 MeOH/H_2O, CV = 20 V): m/z 287.1 (M^{2+}, 100%), 573.1 (M^+, 51%).

2-Mercaptopyrimidine (4′-Chloro-2,2′:6′,2″-terpyridine)platinum(II) Nitrate (7). 4′-Chloro-2,2′:6′,2″-terpyridine (21.4 mg, 0.080 mmol) was platinated as in the synthesis of (**6**). A solution of 2-mercaptopyrimidine (8.9 mg, 0.080 mmol) in methanol/water (1:1, 1.0 mL) was added. The mixture was vortexed and then sonicated for 1.5 h. The mixture was added dropwise to ether/acetone (1:1, 20 mL) to precipitate the complex. The solid was washed with ether/acetone (1:1, 4 × 20 mL) and then dried to yield 2-mercaptopyrimidine (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) nitrate (35.2 mg, 69%) as a dark-purple solid. The product was purified by dissolving the solid in hot methanol/water (1:1) and reprecipitating from ether/acetone (1:1, 25 mL). ^{1}H NMR (400 MHz, D_2O): δ 8.81 (2H, d, J = 4.8 Hz, H6, H6″), 8.49 (2H, s, H3′, H5′), 8.36 (2H, apparent t, J = 7.9 Hz, H4, H4″), 8.25 (2H, d, J = 5.0 Hz, H_x, Hz), 8.23 (2H, d, J = 7.8 Hz, H3, H3″), 7.68 (2H, apparent t, J = 6.7 Hz, H5, H5″), 7.03 (1H,t, J = 5.0 Hz, H_y). ESMS (1:1 MeOH/H_2O, CV = 20 V): m/z 574.1 (M^+, 100%).

2-Mercaptopyridine (4′-Chloro-2,2′:6′,2″-terpyridine)platinum(II) Nitrate (8). 4′-Chloro-2,2′:6′,2″-terpyridine (21.4 mg, 0.080 mmol) was platinated as in the synthesis of **6**. A solution of 2-mercaptopyridine (15 mg, 0.13 mmol) in methanol/water (1:1, 1.0 mL) was added. The mixture was vortexed and then sonicated for 1.5 h. The mixture was added dropwise to ether/acetone (1:1, 20 mL) to precipitate the complex. The solid was washed with ether/acetone (1:1, 4 × 20 mL) and then dried to yield 2-mercaptopyridine (4′-chloro-2,2′:6′,2″-terpyridine)-platinum(II) nitrate (42 mg, 82%) as a yellow solid. The product was purified by dissolving the solid in hot methanol/water (1:1) and reprecipitating from ether/acetone (1:1, 25 mL). ^{1}H NMR (400 MHz, 0.1 M DCl/D_2O): δ_H = 6.90 (1H, t, J = 7 Hz) 7,27 (2H, t, J = 6 Hz, H5/5″), 7.41 (1H, t, J = 8 Hz), 7.70 (2H, t, J = 6 Hz, H4/4″), 7.8−8.0 (4H, m), 8.14 (2H, s, H3′/5′), 8.31 (2H, d, J = 5 Hz, H6/6″). ESMS (1:1 MeOH/H_2O): m/z 572 {M^+, 100%}.

***N,S*-Bis[(2,2′:6′,2″-terpyridine)platinum(II)]-thioacetimine Trinitrate (9).** A solution of silver nitrate (64.6 mg, 0.38 mmol) in acetone/water (4:1, 0.6 mL) was added dropwise to a suspension of diiodo-1,5-cyclooctadieneplatinum(II) (99.6 mg, 0.18 mmol) in acetone/water (4:1, 0.6 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The silver iodide precipitate was discarded. The supernatant was added to a suspension of 2,2′:6′,2″-terpyridine (33.6 mg, 0.144 mmol) in acetonitrile (0.3 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The supernatant was removed and discarded. The

pellet was washed with acetonitrile/ether (1:3, 2 × 2.0 mL) and then dissolved in water (0.75 mL). A solution of thioacetamide (5.41 mg, 0.070 mmol) in water (6.0 mL) was added. The mixture was vortexed and then sonicated for 1 h and then added dropwise to ether/acetone (1:1, 25 mL) to precipitate the *N*,*S*-bis[(2,2′:6′,2″-terpyridine)platinum(II)]-thioacetimine trinitrate as a dark purple-brown solid (52 mg, 67%). The solid was washed with ether/acetone (1:1, 4 × 20 mL) and then dried. The product was purified by dissolving in hot methanol/water (1:1) and reprecipitating with ether/acetone (1:1, 25). ^{1}H NMR (400 MHz, D_2O): δ 8.77,(2H, d, J = 5.5 Hz, H6/6″), 8.42,(1H, t, J = 8.2 Hz, H4′), 8.38 (1H, t, J = 8.2 Hz, H4′), 8.30 (2H, d, J = 5.1 Hz, H6/6″), 8.28−8.20, (4H, m, (2 × H4)/(2 × H4″)), 8.10 (2H, d, J = 8.2 Hz, H3′/5′), 8.05 (2H, d, J = 8.2 Hz, H3′/5′), 8.01 (2H, d, J = 7.7 Hz, H3/3″), 8.00 (2H, d, J = 7.7 Hz, H3/3″), 7.58−7.52 (4H, m, (2 × H5)/(2 × H5″)), 2.87 (3H, s, CH_3). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.60 (1H, s, NH), 8.82 (2H, d, J = 5.6 Hz, H6/6″), 8.55 (1H, t, J = 8.1 Hz, H4′), 8.52 (1H, t, J = 8.1 Hz, H4′) 8.44, (2H, d, J = 5.5 Hz, H6/6″), 8.41 (2H, d, J = 8.1 Hz, H3′/5′), 8.37 (2H, d, J = 8.1 Hz, H3′/5′), 8.34−8.32 (8H, m, 2 × H3, 2 × H3″, 2 × H4, 2 × H4″), 7.70−7.64 (4H, m, 2 × H5, 2 × H5″), 2.95 (3H, s, CH_3). ESMS (1:1 MeOH/H_2O, CV = 20 V): m/z 310.1 (M^{3-}, 100%), 444.1 {[Pt(terpy)−S−Pt(Terpy)]$^{2+}$, 69%).

(2,2′:6′,2″-Terpyridine)platinum(II)-1-thiolato-β-D-glucose Nitrate (10). A solution of silver nitrate (37.0 mg, 0.218 mmol) in acetone/water (4:1, 0.5 mL) was added dropwise to a suspension of diiodo-1,5-cyclooctadieneplatinum(II) (55.0 mg, 0.102 mmol) in acetone/water (4:1, 0.5 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The silver iodide precipitate was discarded. The supernatant was added to a suspension of 2,2′:6′,2″-terpyridine (18.7 mg, 0.080 mmol) in acetonitrile (0.25 mL). The mixture was vortexed and sonicated for a few minutes and then centrifuged. The supernatant was removed and discarded. The pellet was washed with acetonitrile/ether (1:3, 2 × 1.5 mL) and then dissolved in water (0.75 mL). A solution of 1-thio-β-D-glucose (15.3 mg, 0.070 mmol) (Sigma Aldrich Co., Ltd., U.K.) in water (2.0 mL) was added. The mixture was vortexed and then sonicated for 45 min and then added dropwise to ether/acetone (1:1, 20 mL) to precipitate the complex. The solid was washed with ether/acetone (1:1, 4 × 20 mL) and then dried to yield (2,2′:6′,2″-terpyridine)platinum(II) 1-thiolato-β-D-glucose nitrate (38 mg, 79%) as a dark-purple solid. The product was purified by reprecipitation from methanol/ether/acetone (1:4:5, 20 mL). ^{1}H NMR (500 MHz, D_2O): δ 9.11 (2H, br d, J = 3.4 Hz, H6, H6″), 8.32 (1H, t, J = 8.1 Hz, H4′), 8.25 (2H, m, H4, H4″), 8.12 (2H, d, J = 8.1 Hz, H3′, H5′), 8.07 (2H, d, J = 7.9 Hz, H3, H3″), 7.68 (2H, m, H5, H5″), 4.42 (1H, d, J = 8.7 Hz, H_a), 3.71 (1H, d, J = 11.6 Hz, either H_c or H_d), 3.51 (1H, dd, J = 5.5, 11.9 Hz, either H_c or H_d), 3.35−3.23 (4H, m, H_x, H_y, H_b, H_e). ESI (1:1 MeOH/H_2O, CV = 30 V): m/z 623.5 (M^+, 97%).

Samples were judged pure by the absence of nonassignable peaks in high-field ^{1}H NMR spectra and by the molecular ion peak in the ESI mass spectrum with the correct isotope distribution.

Cell Culture Conditions. Primary glioblastoma and HNSCC (head-and-neck squamous cell carcinoma) cells were cultured by dissecting tissue into small pieces of about 1 mm^3 and transferring them into 75 cm^2 plastic tissue culture flasks. Cells were cultured routinely in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics at 37 °C, 5% CO_2, and 95% air in a humidified incubator with medium changes twice a week. After reaching confluency, cells were harvested by a brief incubation with trypsin/EDTA solution (Viralex, PAA, Linz, Austria) and seeded into a fresh 75 cm^2 plastic tissue culture flask. Tumor cells were characterized for their astrocytic or epithelial origin by immunohistochemical detection of tissue-specific markers like GFAP for glioma cells (NCH37, NCH82, and NCH89) and a panel of different cytokeratins for HNSCC cells (HNO97 and HNO199). Only cell cultures exhibiting homogeneous staining for the respective marker were used in this study.

Proliferation Assay. The assay was performed as described by Mueller et al.,[57] using the BrdU Labelling and Detection Kit III from Roche Diagnostics, Mannheim. Cells were seeded in 8 replicas in 96-well plates into RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics, each well receiving 7 × 10^3 cells. After 24 h the synthetic compounds **6** and **9** were added in different concentrations (1, 5, 10, and 20 μM) following one of two application protocols: we used either a single addition on the first day of the 67 h incubation time or three repeated additions in 24 h intervals, again with a total incubation time of 67 h.

BrdU was added to the wells at a final concentration of 100 μM 48 h after the first addition of the compounds. The BrdU-incorporation assay was processed according to the manufacturer's instructions. The mean value of the absorbance of the control samples containing no platinum compounds was defined as 100% of the proliferation rate. All values are means of at least two independent experiments (each comprising eight replicas).

Determination of Enzyme Activities of Cells after Incubation with Compound 6 (See Footnotes of Table 3). The cells were grown, incubated with inhibitor, and harvested as described above. Then they were washed twice in phosphate-buffered saline, taken up in 200 μL of the same buffer, and lysed by sonication. Following a clearing centrifugation, 10 μL samples were used for enzyme assays, the total assay incubation mixture being 100 μL. For TrxR assays, 100 μM *E. coli* thioredoxin was used as a disulfide substrate instead of DTNB. Prior to the addition of thioredoxin the considerable NADPH oxidase activity was measured and subtracted later from the overall activity.

Acknowledgment. The authors thank Irene König, Marina Fischer, and Petra Harwaldt for their excellent technical assistance. The study was supported by the Deutsche Forschungsgemeinschaft (research focus "Selenoproteins" BE 1540/6-1) and the BBSRC (Grant 43/B10436).

Abbreviations

ADF: adult T-cell leukemia-derived factor
BAL: British Anti-Lewisite (2,3-dithiopropanol)
BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine)
BrdU: 5-bromo-2′-deoxyuridine
DTNB: 5,5′-dithiobis(2-nitrobenzoate)
EcTrx: *Escherichia coli* thioredoxin
GBM: glioblastoma multiforme
GFAP: glial fibrillary acidic protein
GSSG: glutathione disulfide
(h)GR: (human) glutathione reductase [EC 1.6.4.2]
HNSCC: head-and-neck squamous cell carcinoma
(h)Trx: (human) thioredoxin
(h)TrxR(s): (human) thioredoxin reductase(s) [EC 1.6.4.5]
NK-lysin: natural killer cell lysin

The following bold arabic numbers have been assigned to the platinum complexes studied here (for structures please see Figure 1).

1: 4-picoline-(2,2′:6′,2″-terpyridine)platinum(II) dinitrate
2: 2-hydroxyethanethiolate-(2,2′:6′,2″-terpyridine)platinum(II) nitrate
3: 2-hydroxyethanethiolate-(4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) nitrate
4: 2-hydroxyethanethiolate-(4′-ethoxy-2,2′:6′,2″-terpyridine)platinum(II) nitrate
5 - *N*,*S*-bis[(4′-chloro-2,2′:6′,2″-terpyridine)platinum(II)]-imidazole-2-thiolate trinitrate
6: (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) pyridine-4-thiolate nitrate
7: (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) pyrimidine-2-thiolate nitrate
8: (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) pyridine-2-thiolate nitrate

9: *N*,*S*-bis(2,2′:6′,2″-terpyridine)platinum(II)-thioacetimine trinitrate

10: (2,2′:6′,2″-terpyridine)platinum(II)-1-thiolato-β-D-glucose nitrate

References

(1) Williams, C. H., Jr. Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase, and mercuric ion reductase—a family of flavoenzyme transhydrogenases. In *Chemistry and Biochemistry of Flavoenzymes*; Müller, F., Ed.; CRC Press: Boca Raton, FL, 1992; Vol III, pp 121−211.

(2) Kanzok, S.; Fechner, A.; Bauer, H.; Ulschmid, J. K.; Müller, H.-M.; Botella-Munoz, J.; Schneuwly, S.; Schirmer, R. H.; Becker, K. Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. *Science* **2001**, *291*, 643−646.

(3) Zhong, L.; Arnér, E. S.; Ljung, J.; Åslund, F.; Holmgren, A. Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *J. Biol. Chem.* **1998**, *273*, 8581−8591.

(4) Arscott, L. D.; Gromer, S.; Schirmer, R. H.; Becker, K.; Williams, C. H. The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3621−3626.

(5) Becker, K.; Gromer, S.; Schirmer, R. H.; Mueller, S. Thioredoxin reductase as a pathophysiological factor and drug target. *Eur. J. Biochem.* **2000**, *267*, 6118−6125.

(6) Mustacich, D.; Powis, G. Thioredoxin reductase. *Biochem. J.* **2000**, *346*, 1−8.

(7) Sun, Q.-A.; Wu, Y.; Zappacosta, F.; Jeang, K.-T.; Lee, B. J.; Hatfield, D. L.; Gladyshev, V. N. Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. *J. Biol. Chem.* **1999,** *274*, 24522−24530.

(8) Holmgren, A.; Arnér, E. S. J.; Aslund, F.; Bjoernstedt, M.; Zhong, L.; Ljung, J.; Nakamura, H.; Nikitovic, D. Redox regulation by the thioredoxin and glutaredoxin system. In *Oxidative stress in cancer, AIDS, and neurodegenerative diseases*; Motagner, L., Olivier, R., Pasquier, C., Eds.; Marcel Dekker: New York, 1998; pp 229−246.

(9) Schirmer, R. H.; Mueller, J. G.; Krauth-Siegel, R. L. Disulfide-reductase inhibitors as chemotherapeutic agents: The design of drugs for trypanosomiasis and malaria. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 141−154.

(10) Tamura, T.; Stadtman, T. C. A new selenoprotein from human lung adenocarcinoma cells: Purification, properties, and thioredoxin reductase activity. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 1006−1011.

(11) Gladyshev, V. N.; Factor, V. M.; Housseau, F.; Hatfield, D. L. Contrasting patterns of regulation of the antioxidant selenoproteins, thioredoxin reductase, and glutathione peroxidase. *Biochem. Biophys. Res. Commun.* **1998**, *251*, 488−493.

(12) Rubartelli, A.; Bajetto, A.; Allavena, G.; Wollman, E.; Sitia, R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **1992**, *267*, 24161−24164.

(13) Rubartelli, A.; Bonifaci, N.; Sitia, R. High rates of thioredoxin secretion correlate with growth arrest in hepatoma cells. *Cancer Res.* **1995**, *55*, 675−680.

(14) Powis, G.; Oblong, J. E.; Gasdaska, P. Y.; Berggren, M.; Hill, S. R.; Kirkpatrick, D. L. The thioredoxin/thioredoxin reductase redox system and control of cell growth. *Oncol. Res.* **1994**, *6*, 539−544.

(15) Gasdaska, P. Y.; Oblong, J. E.; Cotgreave, I. A.; Powis, G. The predicted amino acid sequence of human thioredoxin is identical to that of the autocrine growth factor human adult T-cell derived factor (ADF): thioredoxin mRNA is elevated in some human tumors. *Biochim. Biophys. Acta* **1994**, *1218*, 292−296.

(16) Wakasugi, N.; Tagaya, Y.; Wakasugi, H.; Mitsui, A.; Maeda, M.; Yodoi, J.; Tursz, T. Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein−Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8282−8286.

(17) Becker, K.; Schirmer, R. H. 1,3-Bis(2-chloroethyl)-1-nitrosourea as thiol-carbamoylating agent in biological systems. *Methods Enzymol.* **1995**, *251*, 173−188.

(18) Gromer, S.; Schirmer, R. H.; Becker, K. The 58 kDa mouse selenoprotein is a BCNU-sensitive thioredoxin reductase. *FEBS Lett.* **1997**, *412*, 318−320.

(19) Gromer, S.; Arscott, L. D.; Williams, Ch.; Schirmer, R. H.; Becker, K. Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. *J. Biol. Chem.* **1998**, *273*, 20096−20101.

(20) Fine, H. A. The basis for current treatment recommendations for malignant gliomas. *J. Neurooncol.* **1994**, *20*, 111−120.

(21) Steiner, H.-H.; Herold-Mende, C.; Bonsanto, M.; Geletneky, K.; Kunze, S. Prognosis of brain tumors: epidemiology, survival time and clinical course. *Versicherungsmedizin* **1998**, *5*, 173−178.

(22) Kuratsu, J.; Ushio, Y. Epidemiological study of primary intracranial tumors: a regional survey in Kumamoto Prefecture in the southern part of Japan. *J. Neurosurg.* **1996**, *84*, 946−950.

(23) Kuratsu, J.; Ushio, Y. Epidemiological study of primary intracranial tumours in elderly people. *J. Neurol., Neurosurg. Psychiatry* **1997**, *63*, 116−118.

(24) Preston-Martin, S.; Staples, M.; Farrugia, H.; Giles, G. Primary tumors of the brain, cranial nerves and cranial meninges in Victoria, Australia, 1982−1990: patterns of incidence and survival. *Neuroepidemiology* **1993**, *12*, 270−279.

(25) Walker, A. E.; Robins, M.; Weinfeld, F. D. Epidemiology of brain tumors: the national survey of intracranial neoplasms. *Neurology* **1985**, *35*, 219−226.

(26) Burger, P. C.; Green, S. Patient age, histologic features, and length of survival in patients with glioblastoma multiforme. *Cancer* **1987**, *59*, 1617−1625.

(27) Cohadon, F. Indications for surgery in the management of gliomas. *Adv. Tech. Stand. Neurosurgery* **1990**, *17*, 189−234.

(28) Chambers, R.; Gillespie, Y.; Soroceanu, L.; Andreansky, S.; Chatterjee, S.; Chou, J.; Roizman, B.; Whitley, R. Comparison of genetically engineered herpes simplex viruses for the treatment of brain tumors in a scid mouse model of human malignant gliomas. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1411−1415.

(29) Chamberlain, M. C.; Kormanik, P. A. Practical guidelines for the treatment of malignant gliomas. *West. J. Med.* **1998**, *168*, 114−120.

(30) DeAngelis, L. M.; Burger, P. C.; Green, S. B.; Cairncross, H. G. Malignant glioma: who benefits from adjuvant chemotherapy? *Ann. Neurol.* **1998**, *44*, 691−695.

(31) Wong, E.; Giandomenico, C. M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **1999**, *99*, 2451−2466.

(32) Jennette, K.; Lippard, S. J.; Vassiliades, G. M.; Bauer, W. Metallointercalation reagents. 2-Hydroxyethanethiolato(2,2′,2′-terpyridine)-platinum(II) monocation binds strongly to DNA by intercalation. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3839−3843.

(33) McCoubrey, A.; Latham, H. C.; Cook, P. R.; Rodger, A.; Lowe, G. 4-Picoline-2,2′:6′,2″-terpyridine-platinum(II)—a potent intercalator of DNA. *FEBS Lett.* **1996**, *380*, 73−78.

(34) Comess, K. M.; Lippard, S. J. In *Molecular aspects of anticancer drug−DNA interactions*; Neidle, S., Waring, M., Eds.; The MacMillan Press: New York, 1993; pp 134−168.

(35) Lowe, G.; Droz, A.-S.; Vilaivan, T.; Weaver, G. W.; Tweedale, L.; Pratt, J. M.; Rock, P.; Yardley, V.; Croft, S. L. Cytotoxicity of (2,2′:6′,2″-terpyridine)platinum(II) complexes to *Leishmenia donovani*, *Trypanosoma cruzi*, and *Trypanosoma brucei*. *J. Med. Chem.* **1999**, *42*, 999−1006.

(36) Lowe, G.; Droz, A.-S.; Vilaivan, T.; Weaver, G. W.; Park, J. M.; Pratt, J. M.; Tweedale, L.; Rock, P.; Kelland, L. R. Cytotoxicity of (2,2′:6′,2″-terpyridine)platinum(II) complexes against human ovarian carcinoma. *J. Med. Chem.* **1999**, *42*, 3167−3174.

(37) Reedijk, J. Why does cisplatin reach guanine-N7 with competing S-donor ligands available in the cell? *Chem. Rev.* **1999**, *99*, 2499−2510.

(38) Matsuda, M.; Masutani, H.; Nakamura, H.; Miyajima, S.; Yamauchi, A.; et al. Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependent cytotoxicity on U937 cells. *J. Immunol.* **1991**, *147*, 3837−3841.

(39) Andersson, M.; Gunne, H.; Agerberth, B.; Boman, A.; Bergman, T.; et al. NK-lysin, a novel effector peptide of cytotxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. *EMBO J.* **1995**, *14*, 1615−1625.

(40) Andersson, M.; Holmgren, A.; Spyrou, G. NK-lysin, a disulfide-containing effector peptide of T-lymphocytes, is reduced and inactivated by human thioredoxin reductase. Implication for a protective mechanism against NK-lysin cytotoxicity. *J. Biol. Chem.* **1996**, *271*, 10116−10120.

(41) Schallreuter, K. U.; Gleason, F. K.; Wood, J. M. The mechanism of action of the nitrosourea antitumor drugs on thioredoxin reductase, glutathione reductase and ribonucleotide reductase. *Biochim. Biophys. Acta* **1990**, *1054*, 14−20.

(42) Schallreuter, K. U.; Wenzel, E.; Brassow, F. W.; Berger, J.; Breitbart, E. W.; Teichmann, W. Positive phase II study in the treatment of advanced malignant melanoma with fotemustine. *Cancer Chemother. Pharmacol.* **1991**, *29*, 85−87.

(43) Sasada, T.; Nakamura, H.; Ueda, S.; Sato, N.; Kitaoka, Y.; Gon, Y.; Takabayashi, A.; Spyrou, G.; Holmgren, A.; Yodoi, J. Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to cis-diamminedichloroplatinum (II). *Free Radical Biol. Med.* **1999**, *27*, 504−514.

(44) Yokomizo, A.; Ono, M.; Nanri, H.; Makino, Y.; Ohga, T.; Wada, M.; Okamoto, T.; Yodoi, J.; Kuwano, M.; Kohno, K. Cellular

levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. *Cancer Res.* **1995**, *55*, 4293−4296.
(45) Sun, Y.; Oberley, L. W. Redox regulation of transcriptional activators. *Free Radical Biol. Med.* **1996**, *21*, 335−348.
(46) Rainwater, R.; Parks, D.; Anderson, M. E.; Tegtmeyer, P.; Mann, K. Role of cysteine residues in regulation of P53 function. *Mol. Cell Biol.* **1995**, *15*, 3892−3902.
(47) Matthews, J. R.; Wakasugi, N.; Virelizier, J. L.; Yodoi, J.; Hay, R. T. Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res.* **1992**, *20*, 3821−3830.
(48) Casso, D.; Beach, D. A mutation in a thioredoxin reductase homologue suppresses p53-induced growth inhibition in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **1996**, *252*, 518−529.
(49) Muro, Y.; Ogawa, Y.; Kato, Y.; Hagiwara, M. Autoantibody to thioredoxin reductase in an ovarian cancer patient. *Biochem. Biophys. Res. Commun.* **1998**, *242*, 267−271.
(50) Smith, A. D.; Guidry, C. A.; Morris, V. C.; Levander, O. A. Aurothioglucose inhibits murine thioredoxin reductase activity in vivo. *J. Nutr.* **1999**, *129*, 194−198.
(51) Karplus, P. A.; Krauth-Siegel, R. L.; Schirmer, R. H.; Schulz, G. E. Inhibition of human glutathione reductase by the nitrosourea drugs 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea. A crystallographic analysis. *Eur. J. Biochem.* **1988**, *171*, 193−198.
(52) Davioud-Charvet, E.; Becker, K.; Landry, V.; Gromer, S.; Logé, C.; Sergheraert, C. Synthesis of 5,5′-dithiobis(2-nitrobenzamides) as alternative substrates for trypanothione reductase and thioredoxin reductase: a microtiter colorimetric assay for inhibitor screening. *Anal. Biochem.* **1999**, *268*, 1−8.
(53) Nordhoff, A.; Bücheler, U. S.; Werner, D.; Schirmer, R. H. Folding of the four domains and dimerization are impaired by the Gly446 → Glu exchange in human glutathione reductase. Implications for the design of antiparasitic drugs. *Biochemistry* **1993**, *32*, 4060−4066.
(54) Holmgren, A.; Björnstedt, M. Thioredoxin and thioredoxin reductase. *Methods Enzymol.* **1995**, *252*, 199−208.
(55) Lowe, G.; Vilaivan, T. An improved method for the synthesis of (2,2′:6′,2″-terpyridine)platinum(II) complexes. *J. Chem. Res., Synop.* **1996**, 386−387.
(56) Bonse, S.; Richards, J. M.; Ross, S. A.; Lowe, G.; Krauth-Siegel, R. L. (2,2′:6′,2″-Terpyridine platinum(II) complexes are irreversible inhibitors of *Trypanosoma cruzi* trypanothione reductase but not of human glutathione reductase. *J. Med. Chem.* **2000**, *43*, 4812−4821.
(57) Mueller, M. M.; Herold-Mende, C.; Riede, D.; Steiner, H.-H.; Lange, M.; Fusenig, N. E. Autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor in human gliomas with tumor progression. *Am. J. Pathol.* **1999**, *155*, 1557−1567.

JM001014I