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Original Contribution

Differential genomic and proteomic profiling of glioblastoma cells exposed to terpyridineplatinum(II) complexes

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ABSTRACT

Terpyridineplatinum(II) complexes (TPCs) efficiently inhibit the proliferation of glioblastoma cells *in vitro* and have been tested successfully in a rodent glioblastoma model. Apart from intercalation with DNA, the major mechanism of action of TPCs is a very potent and specific interaction with the human selenoprotein thioredoxin reductase (TrxR). TrxR plays a crucial role in cellular redox homeostasis and protection against oxidative damage. In many malignant cells the thioredoxin system is upregulated, promoting tumor growth and progression. Thus, the thioredoxin system has been proposed to be an attractive target for cancer therapy. This study gives the first comprehensive overview of the effects of TPCs on the transcriptome and proteome of glioblastoma cells. We reveal that under TPC treatment, mechanisms countersteering TrxR inhibition are activated in parallel to DNA-damage-responsive pathways. TPC pressure results in long-term compensatory upregulation of TrxR expression. In parallel, p53 is activated, leading to a range of regulations typical for cell-cycle-arrested cells such as upregulation of CDKN1A, induction of GADD45, inhibition of eIF5A maturation, and reduced phosphorylation of stathmin. We also show that TPCs induce endoplasmic reticulum stress, as they activate the unfolded protein response. This profiling study provides a thorough insight into the spectrum of cellular events resulting from specific TrxR inhibition and characterizes the TPC mode of action.

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The homodimeric human selenoprotein thioredoxin reductase (TrxR; EC 1.8.1.9) plays an important role in cellular redox homeostasis and protection against oxidative damage and mutation. It reduces its substrate thioredoxin (Trx) via an NADPH-dependent reaction. The thioredoxin system regulates or is directly connected with cellular proteins such as ribonucleotide reductase, transcription factors, and peroxiredoxins [1,2]. Through these interactions the TrxR/Trx system contributes to preventing neoplastic as well as oxidative cell-damaging processes [3,4]. In many malignant cells the thioredoxin system has been shown to be upregulated and to promote tumor growth and progression. This is supported by observations that TrxR gene expression coincides with the onset of prostate cancer after castration in animals and that TrxR knockdown leads to a drastic reduction in tumor progression, metastasis, and reduced transcript levels of tumor-related proteins [5]. The specific structural and physicochemical properties of TrxR initiated drug development approaches focusing on the enzyme as an anticancer drug target. Electrophilic substances target the TrxR and reduce its antioxidant activity, which leads to inhibition of cellular proliferation [6–9]. Trx and TrxR1 were shown to be associated with p53 expression in breast cancer and with the regulation of p53-mediated CDKN1A (p21) activation in other tumors [10]. Also inhibition of TrxR using RNA interference leads to an increase in the DNA-binding activity of p53 [11]. Activated p53 affects numerous downstream pathways and can mediate cell cycle arrest and/or apoptosis. From these observations the therapeutic potential of TrxR

Abbreviations: Trx, thioredoxin; TrxR, thioredoxin reductase; TPCs, terpyridineplatinum(II) complexes; I_{23}2N, 4-mercaptopyridine (4′-chloro-2,2′:6′,2″-terpyridine) platinum(II) nitrate; I_{25}2N, 2-mercaptopyridine (2′-chloro-2,2′:6′,2″-terpyridine) platinum(II) nitrate; ds, double stranded; PMF, peptide mass fingerprint; CHCA, cyano-4-hydroxycinnamic acid; SAM, significance analysis of microarrays; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rRNA, ribosomal RNA; DTNB, dithio-bis (2-nitrobenzoic acid); TNB^-, thionitrobenzoate*anion*; GPx, glutathione peroxidase; SOD, superoxide dismutase; GR, glutathione reductase; SPS2, selenophosphate synthetase 2; Bax, BCL2-associated protein X, BCL2, B cell CLL/lymphoma 2 protein; FAS, TNF receptor superfamily, member 6; CCNB1, cyclin B1; CCNE1, cyclin E1; NDRG1, N-myc downstream-regulated gene 1; STMN1, stathmin 1; eIF5A, eukaryotic initiation factor 5A; GSPT1, G1- to S-phase transition protein; PERK, pancreatic ER kinase; ATF, activating transcription factor.

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as a drug target has become evident and can be exploited to develop novel chemotherapeutic strategies.

Glioblastomas are the most aggressive type of gliomas, with a high potential for rapid growth and an early fatal outcome for the patients. There is still no effective therapy against these types of brain tumors, owing to the blood–brain barrier preventing drug delivery or to CNS-related side effects of drugs [12]. Systemic treatment as a single therapy or combined with surgery is urgently needed, as the first-choice drug carmustin is nonselective and unstable in aqueous systems. This nitrosourea drug has TrxR-inhibition properties at micromolar concentrations. Glioblastomas, like many other tumors, show increased levels of redox proteins such as TrxR, Trx, or superoxide dismutase, which are all associated with tumor grading [13]. However, not only the redox machinery but also the consecutive reactions are important for understanding tumor response to novel anticancer drugs. The so-called second-generation platinum complexes with a platinum(II) and (IV) core are subjects of scientific and clinical investigations and have been described as promising drug candidates [14].

Previously treatment with TPCs in a rat C6 glioma model revealed a specific reduction in tumor growth, thus proving the chemotherapeutic value of glioblastoma treatment with these complexes [15]. The molecular events causing the anticancer effect are not yet fully understood but presumably are composed of DNA intercalation effects in parallel with effects of TrxR inhibition. To substantiate these hypotheses we studied TPC-treated glioblastoma cells on the transcriptomic and proteomic levels. A brain- and glioma-specific cDNA microarray chip analysis was used for transcriptome profiling, and a quantitative real-time PCR analysis of selected NCH82 glioblastoma cell transcripts treated with $I_{23}2N$ was performed. On the proteomic level we used two-dimensional gel electrophoresis coupled to mass spectrometry for detecting abundance changes in protein species in the two glioblastoma cell lines NCH82 and NCH89 treated with two different TPCs [16,17].

Our study analyzes the mechanism of chemotherapeutic action of TPCs on the transcriptome and proteome levels and enhances our understanding of the cellular events triggered by TrxR/Trx inhibition.

Materials and methods

Materials

4- and 2-mercaptopyridine (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) nitrate ($I_{23}2N$ and $I_{25}2N$, respectively) were synthesized as described [16,17] and kindly provided by the late Professor Gordon Lowe and Pharminox (Oxford, UK). Purified reagents were, if not otherwise stated, obtained from Bio-Rad, Invitrogen, Merck, Roche, Roth, Serva, and Sigma Chemical Co. (Munich, Karlsruhe, Darmstadt, Mannheim, Heidelberg, and Steinheim, Germany).

Cell culture, cell lysis, and protein solubilization

Primary glioblastoma cells (cell lines NCH89 and NCH82) were cultured as described before [16]. 24 hours after seeding, 15 μM $I_{23}2N$ or $I_{25}2N$ was added to trypsinized cells for 24 h. Control plates without an inhibitor were prepared. Cell number and growth were determined by light microscopy. Cells were harvested by trypsinization and resuspended in an equal volume of RNA/DNA-digestion buffer containing 4 M urea, 10 mM Tris/HCl, 5 mM EDTA, 10 mM $MgSO_4$, 1 mM PMSF, and protease inhibitors at pH 8.0. Cells were ruptured by sonication (3 × 10 s) at 4 °C. RNA and DNA were digested with 1% v/v Benzonase for 30 min at 4 °C. After centrifugation at 14,000 g for 15 min at 4 °C, the supernatant was used for two-dimensional gel electrophoresis (2DE) or immunoblotting or stored at −80 °C.

Two-dimensional gel electrophoresis

2DE was performed according to standardized procedures [18]. One hundred micrograms of each sample to be used for analytical gels was solubilized in a 2D sample buffer, containing 6 M urea, 2 M thiourea, 4% w/v Chaps, 75 mM dithiothreitol (DTT), 0.5% v/v ampholytes, and Orange G in traces. For preparative gels to be analyzed by mass spectrometry 750 μg, of protein extract was applied per IPG strip. A 400 μl-volume of the obtained protein solution was then used to rehydrate 17-cm-long IPG strips, pH 4–9, for 18 h. Isoelectric focusing was carried out with an IPGphor (GE Healthcare, Munich, Germany), by applying a low initial voltage and then a voltage gradient up to 3500 V, with a limiting current of 50 mA/strip. The total product time voltage applied was 80,000 Vh for each strip, and the temperature was set at 20 °C. For the second dimension, the IPG strips were equilibrated for 30 min in a solution of 6 M urea, 2% w/v SDS, 1% DTT, 30% v/v glycerol, 375 mM Tris/HCl (pH 8.8), and traces of bromophenol blue, with gentle agitation. The IPG strips were then laid onto 12% SDS–PAGE gels with an overlay solution containing 1% w/v agarose, 150 mM Tris, 0.2% SDS, pH 6.8. The electrophoretic run was performed in the DALT system (GE Healthcare) in an SDS buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.8, by setting a current of 2 mA for each gel for 1 h, then 30 mA until the end of the run. During the whole run the temperature was set at 15 °C. At the end of the run gels were incubated in a fixing solution containing 40% v/v ethanol and 10% v/v acetic acid for 30 min; after that the gels were stained with silver according to Heukeshoven and Dernick [19]. Preparative gels were stained with a colloidal Coomassie solution containing 0.08% w/v G250, 1% v/v phosphoric acid, 8% w/v ammonium sulfate, and 20% v/v methanol. To generate meaningful statistical data five replicas for each condition were made.

In silico image analysis

Silver-stained 2DE gels were scanned with an ImageScanner (GE Healthcare) and subjected to image analysis with ImageMaster 2D Elite software (GE Healthcare). The spot maps were edited and matched, and the spot volumina (spot area × intensity) of all gels were normalized to remove non-expression-related differences. For normalization the raw volume of each spot in a gel was divided by the total volume of all the spots in the same gel that were also present in all other gels. The comparisons included two glioblastoma cell lines, NCH82 and NCH89, each as control cells and cells treated with $I_{23}2N$ or $I_{25}2N$. A selection of significantly regulated spots (increased or decreased after treatment) was achieved by calculating the ratios of average, normalized spot volumina [(spot area × spot intensity treated)/(spot area × spot intensity control)] and by the use of statistical means (Student's *t* test; Mann–Whitney test).

Protein identification by PMF (peptide mass fingerprint) with MALDI-MS

The spots of interest were automatically excised from the Coomassie-stained gels using an Ettan-Picker robot apparatus (GE Healthcare) and subjected to tryptic digestion as described in Desrivieres *et al.* [20], with minor variations. Briefly, after vacuum-drying, the gel pieces were incubated with modified porcine trypsin (Promega, Mannheim, Germany) at a final concentration of 10 ng/ml in 50 mM ammonium bicarbonate for 16 h at 37 °C. The peptides were extracted twice in 50 μl of acetonitrile/H_2O (50/50), 1% v/v formic acid. The extract was dried and then resuspended with 10 μl of a 0.1% v/v trifluoroacetate solution in acetonitrile/H_2O (50/50). The obtained solutions were loaded onto the MALDI target plate by mixing 0.3 μl of each solution with the

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