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Cisplatin, oxaliplatin, and carboplatin unequally inhibit *in vitro* mRNA translation

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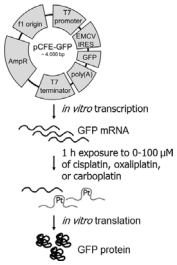
HIGHLIGHTS

- *In vitro* translation system to study effects of Pt-drugs on GFP expression.
- Different effects of platinum cytostatics on mRNA translation.
- mRNA is a functionally relevant target of at least cisplatin.

GRAPHICAL ABSTRACT



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ABSTRACT

DNA is considered the preferential target of platinum containing cytostatics such as cisplatin, oxaliplatin, and carboplatin. Despite profound knowledge on the interaction between platinum drugs and DNA, there is little data on the interaction with mRNA and even less on the potential differences among these antineoplastic agents to inhibit protein synthesis. We therefore established an *in vitro* translation system using *in vitro* transcribed mRNA encoding green fluorescent protein (GFP) to evaluate the effects of exposure of GFP mRNA to 0–100 μM of cisplatin, oxaliplatin, or carboplatin. We additionally investigated the interaction between these drugs and mRNA through evaluation of crossing-points during quantitative real-time polymerase chain reactions. In contrast to oxaliplatin or carboplatin, 100 μM cisplatin significantly increased crossing-points by about 3 cycles ($P < 0.01$) and profoundly attenuated translation of GFP mRNA ($P < 0.05$). Oxaliplatin showed a trend to reduce GFP mRNA translation, whereas carboplatin entirely failed to influence it.

In conclusion, this study for the very first time documents different effects of platinum cytostatics on mRNA translation and demonstrates mRNA to be a functionally relevant target of at least cisplatin.

1. Introduction

Cisplatin, oxaliplatin, and carboplatin are the standard platinum drugs sharing the same basic mechanism of action at their common pharmacological target namely DNA: Upon entering the cell and losing the respective ligands (*e.g.* chloride ions for cisplatin) through substitution with water, the aquated, positively charged platinum drugs interact with nucleophilic N7-sites of purine bases adenine (A) and guanine (G) leading to ApG or GpG inter- and intrastrand crosslinks. Despite this accordance, platinum drugs highly differ in both cancer specific efficacies and toxicities, respectively. For instance, carboplatin is preferred in gynaecological tumours (Fung-Kee-Fung et al., 2007) and mainly exhibits myelosuppression, while oxaliplatin is used for colorectal cancer (Goodwin and Asmis, 2009) and is mainly known for its potential to cause peripheral neuropathy. Cisplatin is administered to treat a broad set of malignancies and exhibits considerable nephro- and ototoxicity (Rabik and Dolan, 2007). The causes for different efficacies and toxicities are not well understood and were initially tried to be attributed to the degree of DNA platination. Respective studies

Abbreviations: DDTC, diethyldithiocarbamate; GFP, green fluorescent protein; IRES, internal ribosome entry site.

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however revealed surprising results demonstrating DNA-adduct formation not to be associated with cytotoxicity of oxaliplatin in colon cancer cells (Arnould et al., 2003) and proving oxaliplatin to cause considerably less DNA platination than cisplatin in Jurkat cells while cytotoxicity was similar (Goodisman et al., 2006). Thus, it is questionable whether DNA is the exclusive target of platinum drugs.

RNA contains the same purine bases as DNA. Thus, the interaction between cisplatin and RNA isoforms is highly probable. Indeed, RNA is considerably platinated (Hostetter et al., 2012; Hägerlöf et al., 2006) and this might have cellular consequences, because RNA-platinum adducts were found in ribosomal RNA (Rijal and Chow, 2009), transfer RNA (Papsai et al., 2008), and messenger RNA (Hostetter et al., 2012). However, the functional consequences of mRNA platination for protein synthesis are poorly investigated so far (Rosenberg and Sato, 1988) and it is unclear whether the known differences among the standard platinum drugs might also be related to their potential to differently inhibit mRNA translation.

We therefore aimed to assess the impact of cisplatin, oxaliplatin, and carboplatin on mRNA translation and thus to characterise mRNA as a functionally relevant pharmacological target of platinum cytostatics.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagles medium (DMEM), L-glutamine and penicillin/streptomycin were purchased from Invitrogen (Karlsruhe, Germany). Foetal calf serum was from PAA Laboratories GmbH (Pasching, Austria), non-essential amino acids from Sigma-Aldrich (Taufkirchen, Germany. RNeasy Mini-Kit was from Qiagen (Hilden, Germany). 96-well microtiter plates were from Nunc (Wiesbaden, Germany). RevertAid™ H Minus First Strand cDNA Synthesis Kit was supplied by Fermentas (St. Leon-Rot, Germany). 1x Absolute QPCR SYBR Green Mix was from Abgene (Hamburg, Germany). NucleoBond Xtra Midi Kit and NucleoSpin Plasmid Kit were purchased from Macherey-Nagel (Düren, Germany). Ambion MEGAscript T7 Kit was from Life Technologies (Carlsbad, USA). Sodium diethyldithiocarbamate was supplied by Sigma-Aldrich (Taufkirchen, Germany). 1-Step Human IVT Kit - mRNA and 1-Step Human IVT Kit - DNA including the pCFE-GFP plasmid were purchased from Pierce Biotechnology (Rockford, USA). Cisplatin, carboplatin and oxaliplatin were supplied by the University Hospital's pharmacy.

2.2. Evaluation of platinum-mRNA interaction by assessment of crossing-point using real-time RT-PCR

The so-called crossing-point (also called 'threshold-point') during PCR reactions indicates the number of amplification cycles needed to exceed background noise for the first time. Hence, lower amounts of accessible mRNA due to platinum-mediated structural disturbances are expected to reduce the efficiency of cDNA synthesis, thus increasing the crossing-points. mRNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany) from LS180 cells (adenocarcinoma cell line; available at ATCC) being cultured at 37 °C and 5% CO_2 in DMEM cell culture medium containing 2 mM glutamine, 0.1 mM non-essential amino acids, 0.2 units/ml penicillin, 0.2 μg/ml streptomycin, and 10% foetal calf serum. Quality and concentration were measured spectrophotometrically using a Nanodrop 1000 (peqlab Biotechnologie GmbH, Erlangen, Germany). 50 μg of RNA were treated with nuclease-free water (control), 0.1 μM, 1 μM, 10 μM, or 100 μM of cisplatin, carboplatin, or oxaliplatin, respectively. Subsequently, platinated RNA was isolated using the RNeasy Mini-Kit again to remove unbound platinum. Quality and concentration were again measured spectrophotometrically and isolated mRNA was stored at −80 °C until analysis. cDNA was synthesised with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions using 200 ng of RNA. Potential crossing-point shifts through mRNA platination were evaluated by real-time RT-PCR with the LightCycler® 480 (Roche Applied Science, Mannheim, Germany) amplifying *ABCC1* (encoding multidrug-resistance associated protein 1), a gene with a long mRNA and thus high probability of being platinated. The 20 μl reaction volume contained 5 μl 1:10 diluted cDNA and 1x Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany). PCR was validated previously (Albermann et al., 2005).

2.3. Establishment of in vitro transcription and in vitro translation

pCFE-GFP is a green fluorescent protein (GFP)-encoding plasmid containing a T7 promoter ensuring efficient *in vitro* transcription by a T7 specific RNA-polymerase (Fig. 1). Due to an EMCV internal ribosome entry site (IRES) within the resulting mRNA cap-independent *in vitro* translation can be performed. For *in vitro* translation, most efficient reaction conditions were initially determined by variation of mRNA amount and reaction times, respectively. Using the 1-Step Human IVT–mRNA Kit, highest protein expression (= highest GFP fluorescence measured *via* Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland) at 485 nm excitation/520 nm emission filter) was finally obtained after 4 h of reaction time at 30 °C using 2 μg mRNA (data not shown). Consequently, all subsequent experiments were performed using 2 μg of mRNA and GFP expression was hourly recorded for up to 4 h.

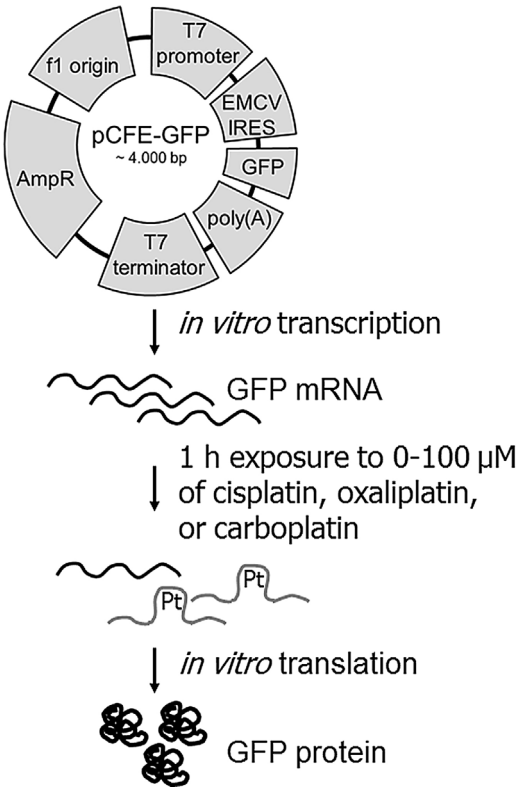

Fig. 1. Scheme depicting the experimental procedure: pCFE-GFP vector was used to generate GFP encoding mRNA being subsequently exposed to 0–100 μM of cisplatin, oxaliplatin, and carboplatin, respectively. After removal of unbound platinum drug, mRNA was *in vitro* translated to GFP protein. Its expression was evaluated through measurement of fluorescence.

2.4. Evaluation of platinum-mRNA interaction by in vitro translation

The pCFE-GFP plasmid was initially amplified in *Escherichia coli* DH5 alpha bacteria and extracted and purified using the NucleoSpin Plasmid Mini Kit (Macherey-Nagel, Düren, Germany). The basic principle of the experimental steps is depicted in Fig. 1.

GFP-encoding mRNA was synthesised using the Ambion MEGAscript T7 Kit (Life Technologies, Carlsbad, USA). Freshly transcribed mRNA was subsequently isolated with a single-step acid guanidium thiocyanate-phenol-chloroform extraction methodology as described before (Chomczynski and Sacchi, 2006). Briefly, after addition of nuclease-free water and ammonium acetate to stop the reaction, an equal volume of phenol chloroform solution containing guanidium thiocyanate (50:49:1) was added. The aqueous phase containing mRNA was transferred and repeatedly treated with an equal volume of chloroform. mRNA was eventually precipitated with 100% ethanol, incubated for 30 min at 4 °C and pelleted through centrifugation. After resuspending in nuclease-free water, quality and concentration were measured spectrophotometrically using a Nanodrop 1000 (peqlab Biotechnologie GmbH, Erlangen, Germany).

For platination 50 μg mRNA were exposed to nuclease-free water (control), 0.1 μM, 1 μM, 10 μM, or 100 μM cisplatin, carboplatin, or oxaliplatin for 1 h at 37 °C, respectively (Fig. 1). Unbound platinum was complexed (Bannister et al., 1979) with a 20-fold molar excess of lipophilic sodium diethyldithiocarbamate (Sigma-Aldrich, Taufkrichen, Germany) and removed during the subsequent isolation of mRNA using the guanidium thiocyanate-phenol-chloroform method as described before. Quality and concentration of mRNA were measured spectrophotometrically using a Nanodrop 1000 (peqlab Biotechnologie GmbH, Erlangen, Germany). *In vitro* translation of GFP-mRNA was performed using the 1-Step Human IVT Kit–mRNA (Pierce Biotechnology, Rockford, USA) using 2 μg of mRNA. This amount has been proved to produce