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RNA is a pro-apoptotic target of cisplatin in cancer cell lines and *C. elegans*

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ABSTRACT

Cisplatin not only targets DNA but also RNA. However, it is largely unknown whether platinated RNA (Pt-RNA) causes apoptosis and thus contributes to the cytotoxic effects of cisplatin. Consequently, cellular RNA was isolated from HepG2 and LS180 cells, exposed to cisplatin, and the resulting Pt-RNA (20 ng Pt/μg RNA) was transfected into these cancer cell lines or used to treat an apoptosis reporter Caenorhabditis elegans (*C. elegans*) strain (MD701, expressing CED-1::GFP). Cellular and molecular effects of Pt-RNA were evaluated by luminogenic caspase 3/7 assays, PCR array analysis, and fluorescence microscopy-based quantification of apoptosis in *C. elegans* gonads. Assuming RNA cross-linking (pseudo double-stranded RNA), the contribution of the Toll-like receptor 3 (TLR3, a sensor of double-stranded RNA) to apoptosis induction in cancer cell lines was investigated by pharmacological TLR3 inhibition and overexpression. In contrast to controls, Pt-RNA significantly enhanced apoptosis in *C. elegans* (2-fold) and in the cancer cell lines (2-fold to 4-fold). TLR3 overexpression significantly enhanced the pro-apoptotic effects of Pt-RNA in HepG2 cells. TLR3 inhibition reduced the pro-apoptotic effects of Pt-RNA and cisplatin, but not of paclitaxel (off-target control). Gene expression analysis showed that Pt-RNA (but not RNA) significantly enhanced the mRNA levels of nuclear factor kappa B subunit 2 and interleukin-8 in HepG2 cells, suggesting that Pt-RNA is a damage-associated molecular pattern that additionally causes pro-inflammatory responses. Together, this data suggests that not only DNA but also cellular RNA is a functionally relevant target of cisplatin, leading to pro-apoptotic and immunogenic effects.

1. Introduction

Cisplatin is an important anti-cancer drug, used to treat a variety of solid tumors [1]. Its mode of action comprises adduct formation at N7 of the DNA purines and 1,2- or 1,3-intra-strand and inter-strand cross--linking of DNA [1,2]. Such DNA lesions cause cell cycle arrest and inhibit the transcription or replication, eventually initiating apoptotic cell death [3]. Given the obvious identical structural prerequisites for platination, RNA is also targeted [4] and has been shown to be quantitatively [5,6] and kinetically preferred over DNA by cisplatin [5,7,8]. Specifically, cisplatin adducts in RNA were found within transfer RNA [8], ribosomal RNA (rRNA) [9], and messenger RNA (mRNA) [5], which can lead to diminished ribosome function and decreased protein synthesis [10,11]. However, it is largely unknown whether platinated RNA (Pt-RNA) also causes apoptosis.

Cisplatin-mediated cross-linking of RNA is expected to lead to pseudo-double-stranded RNA (dsRNA) [7]. In general, dsRNA is a pathogen-associated molecular pattern, indicative for an infection with RNA viruses. Thus, receptors known to recognize and to mediate cellular effects of dsRNA might also be of special relevance for potential pro-apoptotic effects of pseudo-dsRNA originating from cisplatin-mediated RNA cross-linking. For instance, the Toll-like receptor (TLR) 3 is a transmembrane protein of the endosome that recognizes intracellular dsRNA in order to trigger physiological immune responses

Abbreviations: C. elegans, *Caenorhabditis elegans*; DDTC, diethyldithiocarbamate; DsRNA, double-stranded RNA; GFP, green fluorescent protein; PolyI:C, polyinosinic:polycytidylic acid; Pt-RNA, platinated RNA; TLR3, Toll-like receptor 3.

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such as activation of nuclear factor kappa B and subsequent release of cytokines [12]. However, TLR3 is often overexpressed in cancer cells [13–15] and promotes apoptotic cell death when it is activated by dsRNA or the proto-typical TLR3 activator polyinosinic:polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue being frequently used as a positive control for TLR3 engagement [13–15]. Accordingly, the induction of apoptosis by cisplatin-mediated cross-linking of RNA should be mediated at least in part by TLR3.

In summary, this experimental study for the first time evaluated the potential pro-apoptotic effects of Pt-RNA in two cancer cell lines and a *C. elegans* apoptosis reporter model. The relevance of TLR3 for Pt-RNA-mediated apoptosis was assessed by pharmacological TLR3 inhibition and TLR3 overexpression experiments. Large-scale gene expression analysis of Pt-RNA-transfected HepG2 cells was performed using a Fluidigm dynamic PCR array.

2. Materials & methods

2.1. Materials

Cell culture consumables were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany). Diethyldithiocarbamate (DDTC), Dulbecco's Modified Eagle Medium, Fluoroshield™ mounting medium, non-essential amino acids solution, paclitaxel, penicillin-streptomycin, fetal calf serum (FCS), and RPMI 1640 medium were purchased from Sigma-Aldrich (Taufkirchen, Germany). Poly(I:C) High Molecular Weight was from Invivogen (Toulouse, France). The Ulysis™ Alexa Fluor™ 488 Nucleic Acid Labeling Kit was from Thermo Fisher (Dreieich, Germany). The TLR3/dsRNA complex inhibitor was purchased from AppliChem (Darmstadt, Germany). The pCMV-TLR3-C-GFPSpark vector was purchased from Sino Biological Inc. (Beijing, China). Xfect® RNA transfection reagent was from Takara BIO (Kusatsu, Japan). The Caspase-Glo® 3/7 Assay System, Fugene® and the GloMax® microplate reader were from Promega (Mannheim, Germany). Chloroform was obtained from Merck & Co (Darmstadt, Germany). RNeasy RNA isolation kits and Qiazol® were from Qiagen (Heiden, Germany). Agarose was from Axon Labortechnik GmbH (Kaiserslautern, Germany). Micro Bio-Spin 30 Columns were from Bio-Rad (Feldkirchen, Germany). Cisplatin was supplied by the Heidelberg University Hospital's pharmacy.

2.2. Cell lines

LS180 cells and HepG2 cells are available at American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in a humidified incubator (37 °C, 5% CO2) and grown in Dulbecco's Modified Eagle Medium (LS180 cells) or RPMI 1640 medium (HepG2 cells), each supplemented with 10% FCS and penicillin (100 U/mL)-streptomycin (0.1 mg/mL). LS180 medium also contained non-essential amino acids. Identity of both cell lines was confirmed by the PCR-single-locus-technology-based Cell line Authentication Test, provided by Eurofins Genomics Europe Applied Genomics GmbH (Ebersberg, Germany).

2.3. Platination of RNA

Cellular RNA was isolated with the RNeasy Midi Kit according to manufacturer's instructions. Isolated RNA was exposed to 1 mM cisplatin for 1 h at 37 °C. Purification of Pt-RNA was performed according to the method of Chomczynski & Sacchi [16], with some modifications. In brief, a 20-fold molar excess (20 mM) of sodium diethyldithiocarbamate (DDTC) was added and incubated for 1 h at 37 °C in order to chelate free, unbound cisplatin [17,18]. All subsequent indications of volumes refer to this starting volume (= 1.0 vol). Two volumes of a 1:1 chloroform/Qiazol®-solution was added. After mixing, the upper RNA-containing phase was transferred into a new tube. To increase the RNA yield, two volumes of distilled water were added to the lower remaining phase, mixed, and the upper phase was again transferred into the same new tube. Residues of DDTC and Qiazol® in the RNA phase were removed by adding two volumes of chloroform, mixed, and the upper RNA phase was transferred into a new tube. This step was repeated once. In order to precipitate the RNA, four volumes of ice-cold ethanol were added, incubated overnight at −20 °C, and centrifuged for 15 min at 4 °C and 20,000 g. Subsequently, the RNA pellet was thoroughly washed three times with ice-cold ethanol (to remove potential residues of cisplatin), air-dried at room temperature (RT), and dissolved in an adequate volume of RNase-free water. RNA concentrations and purities were measured photometrically and RNA integrities were demonstrated by 1.5% agarose gel electrophoresis.

Platination of RNA was confirmed and quantified using a PinAAcle 900Z (PerkinElmer) graphite furnace atom absorption spectrometer, measuring the background-corrected peak area of the Pt absorption at 265.94 nm after the injection of 20 μL sample volume. The following protocol was used: Thirty s at 110 °C, 30 s at 130 °C, 20 s at 1300 °C, 5 s at 2200 °C, and 3 s at 2450 °C.

Validity of the assay was ensured with a weighted linear calibration curve of five aqueous calibration samples (100–4000 μg Pt/L), three aqueous quality controls (700, 1500, 2500 μg Pt/L), and a RNA-solution quality control (1600 μg RNA/mL), spiked to 1500 μg Pt/L, using a certified stock Pt standard (PerkinElmer).

2.4. Establishment of Pt-RNA transfection methodology

To confirm the feasibility of Pt-RNA transfection and establish a transfection protocol, extracted total RNA was treated with an Alexa Fluor™ 488-labeled cisplatin derivative according to the manufacturer's instructions. This approach combines the pharmacology of Pt-RNA (cisplatin adducts at RNA) with the possibility to track its transfer into cells given the fluorescent moiety. In brief, isolated RNA was precipitated by adding 1/10 of volume of sodium acetate (3 M, pH 5.2) and two volumes of ice-cold pure ethanol and subsequent incubation at −80 °C for 30 min. The RNA pellet was centrifuged for 15 min at 20,000 g, washed with ice-cold pure ethanol, and eventually air-dried. After adding labeling buffer and the Alexa Fluor™ 488-labeled platinum compound, the mixture was incubated for 10 min at 90 °C. The labeled RNA was purified using polyacrylamide gel-based Micro Bio-Spin 30 Columns from Bio-Rad. Columns were equilibrated by allowing the excess packing buffer to drain by gravity to the top of the gel bed first and then spinning the column for 2 min at 1000 g. After loading the sample, the column was centrifuged for 4 min at 1000 g. Transfection of fluorescently labeled Pt-RNA was performed as described below. Controls comprised non-transfected cells and 'transfection' of the Alexa Fluor™ 488-labeled platinum compound alone (without conjugated RNA). Nuclei were counterstained with DAPI (contained in the mounting medium). Pictures were taken by an Olympus IX51 microscope, equipped with a XM10 Camera (Olympus, Hamburg, Germany).

2.5. Transfection of Pt-RNA

The prior experiments with Alexa Fluor™ 488-labeled RNA yielded a transfection approach that was subsequently used for Pt-RNA transfections: LS180 or HepG2 cells (1.5×10^4 in 100 μL cell culture medium) were seeded in white-wall, transparent-bottom 96-well microplates. After 24 h of cultivation, cells were exposed to serum-free culture medium for 4 h. Cells were then transfected with their Pt-RNA or untreated RNA using the Xfect® Takara RNA transfection kit according to the manufacturer's protocol. Poly(I:C) was transfected using FugeneHD from Promega according to the manufacturer's instructions. To exclude potential confounder effects mediated by DDTC or DDTC-complexed cisplatin, control experiments with these compounds were conducted. After 3 h of exposure to the respective samples or control compounds, cells were put on complete cell culture media. Caspase-Glo® 3/7 activity assays were performed 18 h after transfection. All experiments were

performed in triplicates.

2.6. Caspase activity assay

After 18 h of transfection, the Caspase-Glo® 3/7 assay was performed according to manufacturer's instructions. The provided Caspase-Glo® 3/7 reagent contains a pro-luminescent caspase 3/7 substrate that is cleaved to release aminoluciferin, a substrate of luciferase. The Caspase-Glo® 3/7 reagent was added to the cells and incubated on a rotatory shaker for 30 min at room temperature. During this time, activated caspases cleave the substrate and thus cause bright luminescence, an indicator of apoptosis. Luminescence was recorded with a Glomax® luminometer from Promega.

2.7. Apoptosis mediated by cisplatin or paclitaxel, modulated by TLR3 inhibition

To evaluate the effects of the TLR3 inhibitor on apoptosis that is mediated by cisplatin or paclitaxel, LS180 cells or HepG2 cells were seeded and exposed to medium containing 0 μM, 10 μM, or 50 μM of a specific TLR3 inhibitor [19]. After 4 h of exposure, cells were treated for 20 h with different concentrations of cisplatin (LS180 cells: 30 μM, 50 μM; HepG2: 8 μM, 10 μM) or paclitaxel (LS180: 30 nM, 50 nM; HepG2: 10 nM; 30 nM), reflecting concentrations that cause sub-maximum or maximum caspase 3/7 activation. All experiments were performed in triplicates.

2.8. Overexpression of TLR3 and its effect on Pt-RNA-mediated apoptosis

The relevance of TLR3 for Pt-RNA-mediated apoptosis was evaluated by its overexpression in LS180 cells and HepG2 cells. Therefore, 1.5×10^4 cells were seeded in 96-well plates with transparent bottom and incubated for 4 h. Afterwards, cells were set to serum-free DMEM for 4 h. Then, cells were transfected with the GFPSpark-tagged TLR3 plasmid using the Fugene® HD transfection reagent according to manufacturer's instructions. The next day, successful transfection of the cells was evaluated by fluorescence microscopy showing bright green fluorescence, mediated by the green fluorescent protein (GFP) that is tagged to the TLR3 protein. Cells were subsequently re-set to serum-free DMEM medium and transfected with RNA, Pt-RNA (using Xfect® Takara RNA transfection kit), or poly(I:C) (using the Fugene® HD transfection reagent). Caspase-Glo® 3/7 assays were conducted as described above.

2.9. Evaluation of apoptosis in C. elegans

To confirm Pt-RNA-mediated apoptosis in a multicellular organism, the CED-1::GFP reporter Caenorhabditis elegans (*C. elegans*) strain was used [20–22]. Apoptotic cell death in mitotically active germ cells of this worm leads to exposure of phosphatidylserine on the outer leaflet of the plasma membrane. This 'eat-me' signal triggers phagocytosis of the dying cells by engulfing gonadal sheath cells, that express CED-1, a phosphatidylserine-binding protein. By tagging CED-1 with GFP, compaction of CED-1 in apoptotic gonad arms can eventually be localized and quantified [22]. The experiments complied with the ARRIVE guidelines and were conducted in accordance with the U.K. Animals (Scientific Procedures) Act from 1986 and its associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Research Council's Guide for the Care and Use of Laboratory Animals. The respective reporter strain (MD701 bcIs39 [lim-7p::ced-1::GFP + lin-15 (+)]) was cultured, maintained, synchronized, and treated according to previously published standard procedures [23]. In brief, larval stage 4 animals were initially washed and exposed for 24 h at 20 °C to soaking buffer that contained RNA (50 μg/mL), Pt-RNA (50 μg/mL at 18.6 ng Pt/μg RNA), or cisplatin (50 μM) used as a pro-apoptotic positive control. After treatment, the animals were set to drug-free agar plates (recovery, apoptosis induction). Finally, the number of apoptotic germ cell corpses in the gonads (anterior or posterior) was counted in anaesthetized (25 mM sodium azide) animals using a BX43 fluorescence microscope (Olympus).

2.10. Gene expression profiling

To profile gene expression upon treatment of HepG2 cells with RNA, Pt-RNA, or cisplatin (10 μM, 24 h), high-throughput RT-qPCR was performed according to our previously published protocol using a Fluidigm dynamic array on a BioMark™ system [24]. This method enables the simultaneous expression analysis of 95 genes. Following RNA isolation of treated cells using the NucleoSpin® RNA Plus Kit (Machery-Nagel, Düren, Germany), 1 μg of total RNA was reverse transcribed into complementary DNA (cDNA) with the qScript™ cDNA Synthesis Kit (Quanta, Beverly, USA) according to the manufacturer's instructions. Specific target gene amplification (STA) was carried out in order to obtain sufficient amounts of templates of the target genes for the subsequent high-throughput qPCR, followed by enzymatic digestion with E. coli exonuclease I (Exo I) (New England BioLabs, Frankfurt am Main, Germany) to remove unincorporated primers and desoxy-nucleotide triphosphates. For high-throughput qPCR, samples and primers were loaded onto a 96.96 Dynamic Array IFC (integrated fluidic circuit) (Fluidigm, San Francisco, USA), which was transferred into the BioMark™ HD System (Fluidigm, San Francisco, USA). qPCR and melting curve analyses were performed as described previously [24]. Evaluation and data analysis were accomplished with Fluidigm Real-Time PCR Analysis and with the GenEx software. For normalization, five reference genes were used (beta actin, beta2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, glucuronidase-beta, and hypoxanthine phosphoribosyltransferase 1). Changes of the transcript levels of the target genes were displayed as fold change compared to the untreated control group by calculating relative quantities corresponding to the ΔΔCt method [25]. The alterations of gene expression are shown as log2-fold changes with a value of 0 for the untreated control. This depiction was chosen as it enables a clear presentation of both induction (with the value 1 for two-fold enhancement) and repression (with the value −1 for reduction to 50%) of transcription.

2.11. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 7.02 software (GraphPad Software, San Diego, USA). The effect of the treatments on the apoptosis rate in LS180 cells, HepG2, or *C. elegans* was evaluated by ANOVA with Dunnett's post-hoc test. The effect of cisplatin on apoptosis rate in *C. elegans* was evaluated by Student's T-test. The impact of TLR3 inhibition on the caspase 3/7 activities in LS180 cells and HepG2 cells, treated with RNA, Pt-RNA, or poly(I:C) was evaluated by two-way ANOVA with Dunnett's post-hoc test. Two-way ANOVA with Sidak's post-hoc test was used to examine the effect of TLR3 overexpression on Pt-RNA-mediated enhancement of caspase 3/7 activities in LS180 cells and HepG2 cells that were either transfected with the GFPSpark-tagged TLR3 plasmid or left not transfected and subsequently transfected with Pt-RNA or left untreated. Gene expression levels in treated HepG2 cells (cisplatin, RNA, Pt-RNA) compared to untreated HepG2 cells were evaluated by Brown-Forsythe and Welch ANOVA tests, followed by Dunnett's post-hoc test. A P-value < 0.05 was considered significant.

3. Results

3.1. RNA can be platinated by cisplatin and be transfected into cancer cell lines

RNA isolated from LS180 cells or HepG2 has been exposed to cisplatin. The resulting Pt-RNA solutions had RNA concentrations of 1600 μg/mL each. RNA platination was demonstrated and quantified by

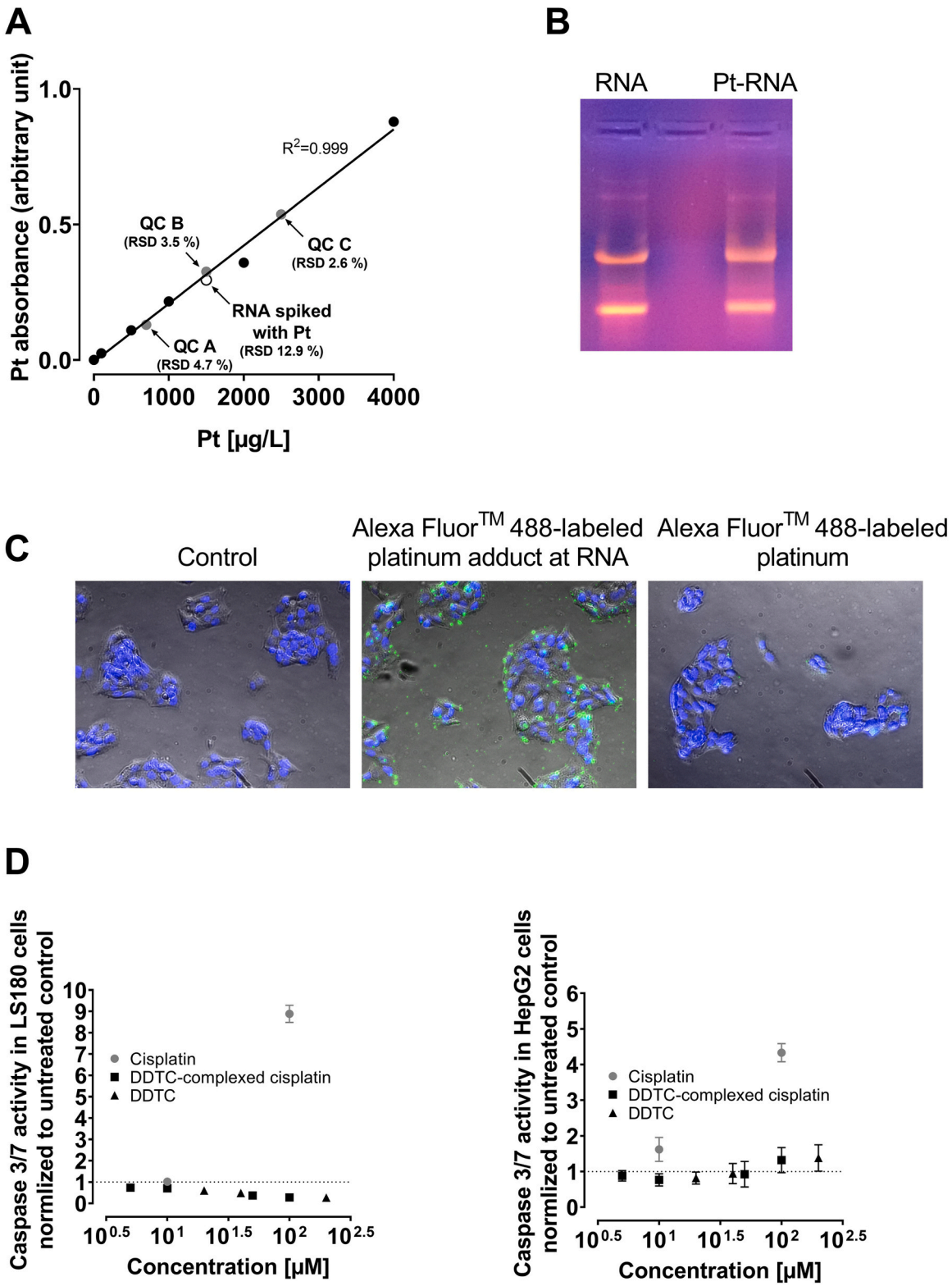

Fig. 1. : Characterization of platinated RNA (Pt-RNA), its transfectability and exclusion of pro-apoptotic by-stander effects of platination process compounds. (A) Calibration curve for Pt quantification, using calibration solutions (black circles), quality controls (gray circles), and an RNA-solution quality control (white circle). Relative standard deviation (RSD) values from three replicate measurements of the same sample are given in parentheses. (B) RNA and Pt-RNA were run on a 1.5% agarose gel (containing ethidiumbromide), showing intact 18 S and 28 S rRNA bands. (C) HepG2 cells were left untreated (left), transfected with RNA that was treated with Alexa Fluor™ 488-labeled platinum compound (middle), or 'transfected' with this fluorescent cisplatin derivative alone (without attachment to RNA, right). (D) LS180 cells and HepG2 cells were treated with diethyldithiocarbamate (DDTC, chelator of cisplatin), DDTC-complexed cisplatin, or cisplatin (positive control), and evaluated for relative caspase 3/7 activity. Data are shown as mean ± S.D. of triplicates.

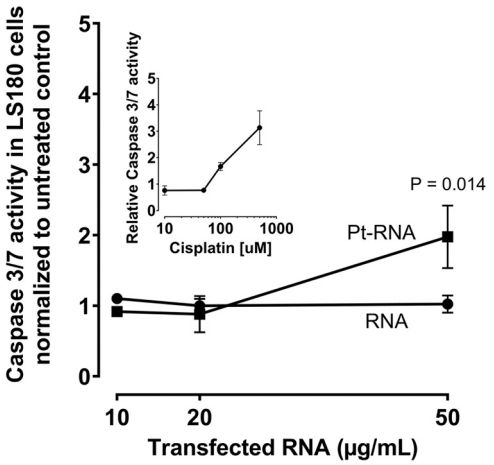

Fig. 2. : Effect of different concentrations of RNA or platinated RNA (Pt-RNA) on caspase 3/7 activity in LS180 cells, normalized to untreated control. Data are shown as mean ± S.D. of triplicates. Statistical significance was evaluated by ANOVA with Dunnett's post-hoc test. Insert: Effects of cisplatin on caspase 3/7 activity, normalized to untreated control. Data are shown as mean ± S.D. of triplicates.

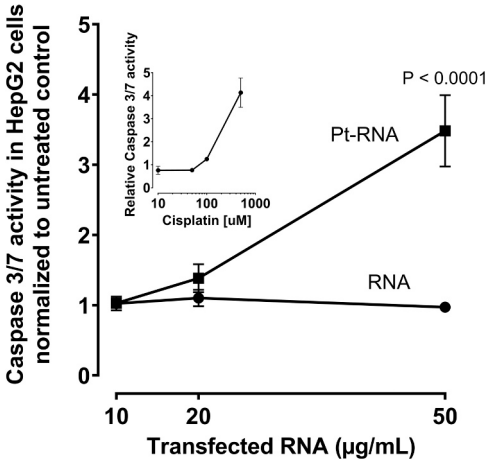

Fig. 3. Effect of different concentrations of RNA or platinated RNA (Pt-RNA) on caspase 3/7 activity in HepG2 cells, normalized to untreated control. Data are shown as mean ± S.D. of triplicates. Insert: Effects of cisplatin on caspase 3/7 activity, normalized to untreated control. Data are shown as mean ± S.D. of triplicates.

graphite furnace atomic absorption spectrometry (weighted linear calibration curve R square value 0.999; Fig. 1A): The Pt-RNA solution from LS180 cells had a Pt content of 34.5 µg/mL, whereas the Pt-RNA solution from HepG2 cells had a Pt concentration of 29.8 µg/mL, corresponding to a platination degree of 21.5 ng Pt/µg RNA (LS180 cells) and 18.6 ng Pt/µg RNA (HepG2 cells). Absorbance measurements at 280 nm (proteins) and 230 nm (contaminations by salts, phenols, etc.) showed high purity of Pt-RNA samples. Agarose gel electrophoresis of samples showed distinct 18 S and 28 S rRNA bands (Fig. 1B), confirming structural integrity of Pt-RNA. RNA treated with Alexa Fluor™ 488-labeled platinum was transfected into HepG2 cells, which subsequently showed bright green fluorescence (Fig. 1C). In contrast, control cells or cells 'transfected' with the Alexa Fluor™ 488-labeled platinum compound alone did not show fluorescence, demonstrating transfectability of RNA with platinum adducts. Because observed pro-apoptotic effects of Pt-RNA could theoretically be mediated by residual DDTC or DDTC-complexed cisplatin in the Pt-RNA sample, control experiments were conducted. DDTC or DDTC-complexed cisplatin did not elevate caspase 3/7 activities, while cisplatin (100 µM) as a positive control enhanced caspase 3/7 activities (Fig. 1D).

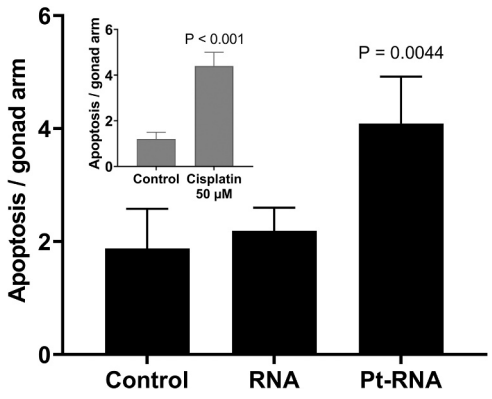

Fig. 4. : Pro-apoptotic effects of platinated RNA (Pt-RNA) in MD701 *C. elegans*, expressing the CED-1::GFP apoptosis reporter. Animals were exposed to RNA (50 µg/mL), Pt-RNA (50 µg/mL), or left untreated. Apoptotic rings in the gonad arms were quantified by fluorescence microscopy. Data are shown as mean ± S.D. of triplicates or quadruplets, comprising 48–82 animals in total. Insert: Effect of cisplatin (50 µM) on apoptosis rate. Data are shown as mean ± S.D. of one control experiments comprising 13–15 animals.

3.2. Pt-RNA causes apoptosis in cancer cell lines

Pt-RNA transfected in LS180 cells ($P = 0.014$) or HepG2 cells ($P < 0.0001$) enhanced caspase 3/7 activities at 50 µg/mL (Fig. 2, Fig. 3). In contrast, 10 µg/mL and 20 µg/mL had no effects. To estimate effect sizes of 50 µg/mL Pt-RNA with the degree of apoptosis induction mediated by cisplatin, both cell lines were exposed to cisplatin in separate control experiments. About 100 µM to 500 µM cisplatin was needed for comparable caspase 3/7 enhancements in these cell lines.

3.3. Pt-RNA causes apoptosis in C. elegans

The used *C. elegans* reporter strain showed some background apoptosis (approx. two apoptotic rings per gonad), that was not enhanced by RNA (50 µg/mL). In contrast, exposing the animals to Pt-RNA (50 µg/mL; 18.6 ng Pt/µg RNA) doubled the apoptosis rate (approx. four apoptotic rings per gonad; $P = 0.0044$) (Fig. 4). In a separate control experiment, 50 µM cisplatin enhanced apoptosis comparably (approx. four apoptotic rings per gonad arm; $P < 0.001$).

3.4. TLR3 contributes to the pro-apoptotic effects of Pt-RNA in cancer cell lines

LS180 cells and HepG2 cells were transfected with RNA, Pt-RNA, or poly(I:C). Concurrent treatments with the TLR3 inhibitor (10 µM, 50 µM) significantly diminished the effects of Pt-RNA in LS180 cells and HepG2 cells (Fig. 5). Likewise, the pro-apoptotic effects of the TLR3 activator poly(I:C) were also reduced in LS180 cells (50 µM) and HepG2 cells (10 µM, 50 µM). In contrast, effects of RNA were not affected.

To further evaluate the relevance of TLR3 for Pt-RNA-mediated apoptosis initiation, a TLR3-encoding plasmid was transfected into the two cell lines, that were subsequently also transfected with Pt-RNA. The apoptosis rate in TLR3-transfected and Pt-RNA-treated LS180 cells was 2-fold higher than in control cells ($P = 0.022$). However, the overexpression of TLR3 only slightly ($P = 0.055$) influenced the observed pro-apoptotic effects of Pt-RNA ($P = 0.012$). In HepG2 cells, apoptosis rate in TLR3-transfected and Pt-RNA-treated cells was 4-fold higher than in control cells ($P < 0.0001$), and the TLR3 transfection ($P = 0.0001$) contributed to this Pt-RNA-mediated effect ($P = 0.0004$), showing a significant interaction of these variables ($P = 0.014$) (Fig. 6).

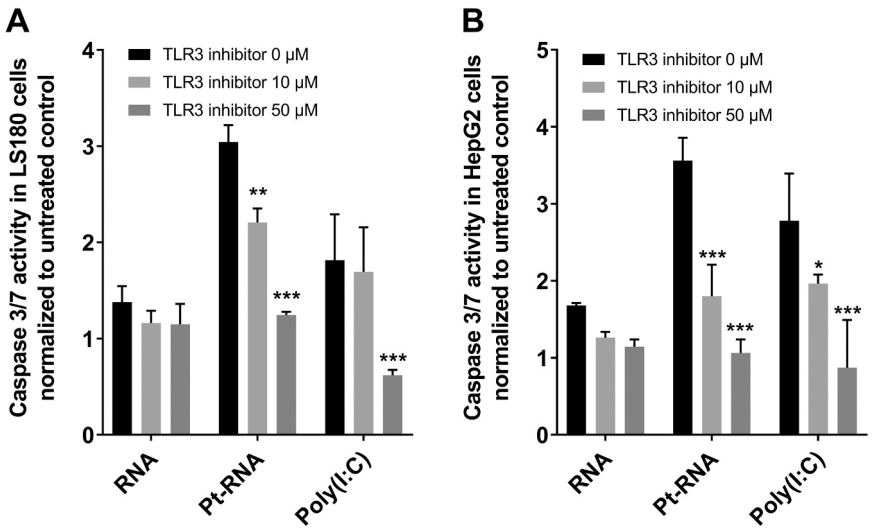

Fig. 5. : Impact of TLR3 inhibition on the caspase 3/7 activity in LS180 cells (A) and HepG2 cells (B), transfected with 50 μg/mL of RNA, platinated RNA (Pt-RNA), or poly(I:C), normalized to untreated control. Data are shown as mean ± S.D. of triplicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

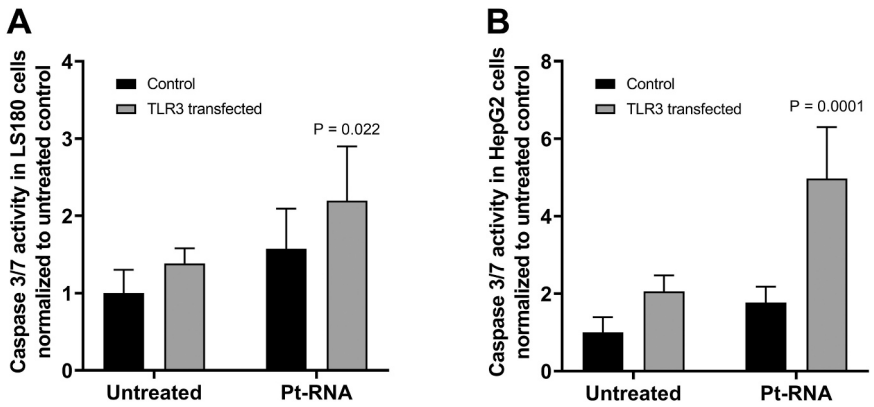

Fig. 6. : Impact of TLR3 overexpression on platinated RNA (Pt-RNA)-mediated enhancement of caspase 3/7 activity in LS180 cells (A) and HepG2 cells (B). Cells were transfected with the GFPSpark-tagged TLR3 plasmid or left non-transfected (control) and subsequently transfected with Pt-RNA or left untreated. Data are shown as mean ± S.D. of quadruplets.

3.5. Pt-RNA induces pro-inflammatory gene signatures in HepG2 cells

Because Pt-RNA had most pronounced effects in HepG2 cells, this cell line was profiled for gene expression after its transfection with RNA or Pt-RNA (50 μg/mL; 18.6 ng Pt/μg RNA). Cisplatin (10 μM, 24 h) exposure was used as a positive control and induced many genes implicated in apoptosis initiation, cell cycle control, and epigenetic regulation (Fig. 7; Table S1). Nuclear factor kappa B subunit 2 (cisplatin: log2 difference 2.23, $P = 0.01$; Pt-RNA: log2 difference 0.25, $P = 0.026$) and interleukin-8 (cisplatin: log2 difference 3.48, $P = 00.012$; Pt-RNA: log2 difference 1.38, $P = 0.013$) were significantly induced by both cisplatin and Pt-RNA. In contrast, RNA had no effects.

3.6. TLR3 contributes to the pro-apoptotic effects of cisplatin, but not paclitaxel

To eventually evaluate whether TLR3 also contributes to the pro-apoptotic effects of cisplatin (and not only Pt-RNA), LS180 and HepG2 cells were exposed to cisplatin concentrations shown to cause sub-maximum or maximum increases in caspase 3/7 (Fig. 8A). Fifty μM of the TLR3 inhibitor significantly reduced the pro-apoptotic effects of 30 μM cisplatin in LS180 cells, whereas the same inhibitor concentration reduced the pro-apoptotic effects of 8 μM and 10 μM cisplatin in HepG2 cells (Fig. 8B). To exclude off-target effects of the TLR3 inhibitor, the same experimental set-up was used for paclitaxel. However, none of the TLR3 inhibitor concentrations reduced the caspase 3/7 enhancements elicited by paclitaxel (30 μM, 50 μM in LS180 cells; 10 μM, 30 μM in HepG2 cells, representing paclitaxel concentration causing sub-maximum or maximum apoptosis stimulation, Fig. 9A) (Fig. 9B).

4. Discussion

In view of the numerous functions of RNA species, it can be assumed that cisplatin adducts on this important nucleic acid may be of biological significance. After exposure of cells to cisplatin, platination of various RNA species has been documented [4], and the structural interaction of cisplatin with cellular RNA molecules has already been investigated down to atomic resolution [11]. Surprisingly, the functional consequences of cisplatin adducts on cellular RNA are not well investigated and it is unknown whether they exert pro-apoptotic effects, although their potential contribution to the antineoplastic cisplatin efficacy has been hypothesized previously [26]. Because it is not possible in cell experiments to allow cisplatin to selectively act on the RNA without also damaging the DNA, an experimental trick was used. RNA was isolated, platinated in a cell-free environment, and then used to investigate its pro-apoptotic effect using various experimental approaches. Several

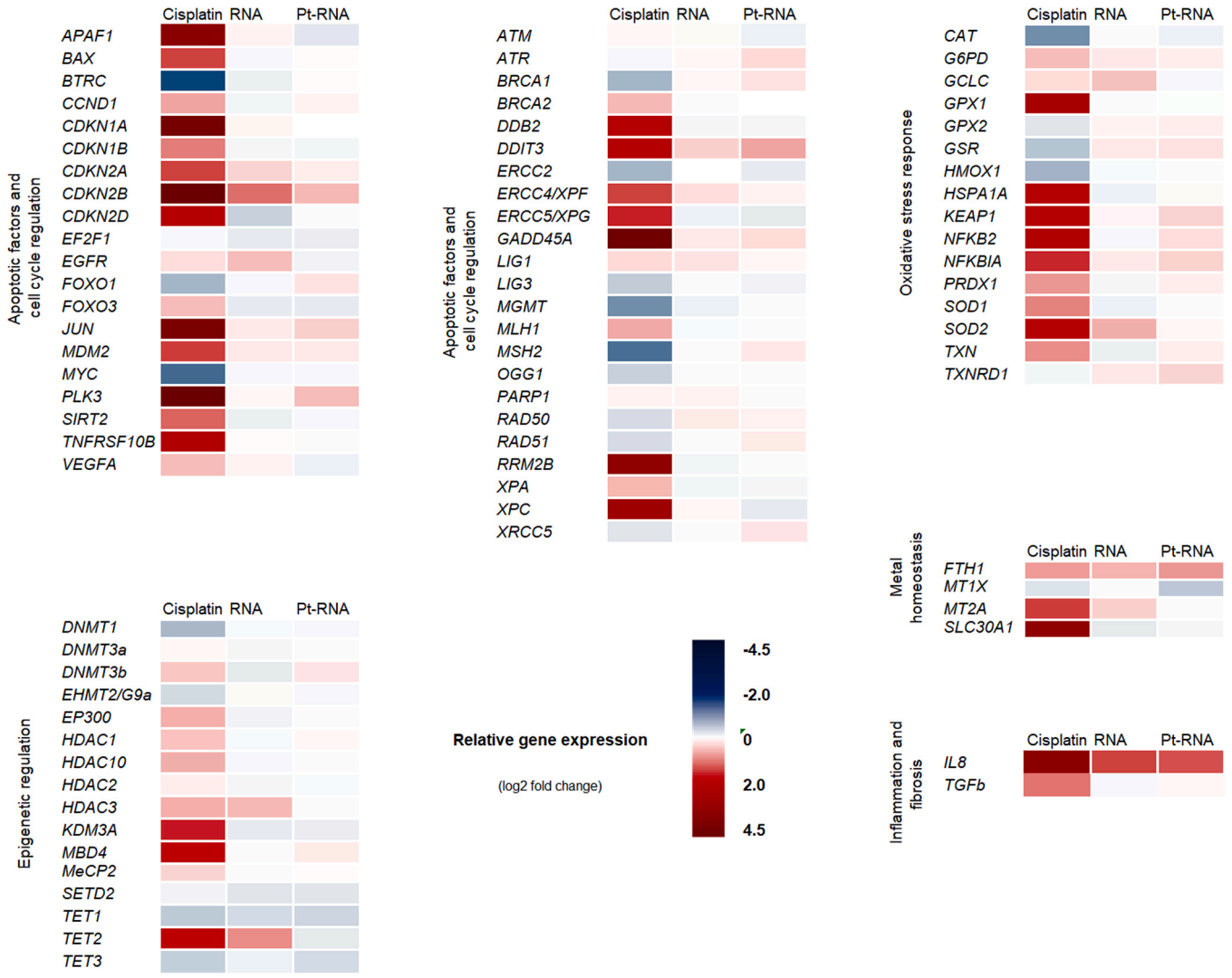

Fig. 7. : Heatmap of gene expression profiling of HepG2 cells transfected with 50 μg/mL of RNA or platinated RNA (Pt-RNA), or treated with 10 μM cisplatin for 24 h. Shown are the log2 mean values of four independent experiments, normalized to the untreated control set to 0.

control experiments underline this finding. First, untreated RNA and the compounds used during the platination process (e.g., DDTC) did not cause apoptosis. Second, before using the aqueous Pt-RNA solutions, the pelleted Pt-RNA had been washed three times with ice-cold pure ethanol to remove potential free residual cisplatin that might cause DNA damage upon transfection. Third, Pt-RNA-mediated apoptosis was observed independently in two unrelated cancer cell lines and in an *in vivo* model. Together, this suggests that the observed effects truly originate from Pt-RNA. Interestingly, the effect sizes of Pt-RNA were comparable to treatments with cisplatin concentrations in the high μM range, suggesting that this mechanism may well contribute to the clinical effects.

Besides describing the pro-apoptotic effect of Pt-RNA, a closer look into the mode of action was needed. Assuming generation of pseudo-dsRNA by RNA cross-linking, the relevance of TLR3 was evaluated. First, TLR3 overexpression experiments revealed a contribution of TLR3 to Pt-RNA-mediated apoptosis at least in HepG2 cells. Second, TLR3 targeting with the competitive, non-toxic, and TLR3-specific inhibitor [19] decreased the pro-apoptotic effects of Pt-RNA and poly(I:C), an established TLR3 activator [14,15]. Taken together, these findings strongly suggest that TLR3 contributes to apoptosis induction by Pt-RNA. To assess whether TLR3 also contributes to the toxicity of cisplatin [15], cells were exposed to sub-maximum and maximum pro-apoptotic cisplatin concentrations in the presence and absence of the TLR3 inhibitor. In LS180 cells, apoptosis initiation was unaffected by the high cisplatin concentration and the reason for that likely is that DNA platination and its downstream effects predominate at maximum cisplatin effects (Emax). In contrast, apoptosis induction in LS180 cells mediated by the moderate cisplatin concentrations (approx. at EC50) was clearly lowered. In HepG2 cells, both cisplatin concentrations led to weaker inductions of apoptosis when the TLR3 inhibitor was administered concurrently. That this is a specific effect of TLR3 inhibition and not a general anti-apoptotic effect is suggested by the fact that apoptosis induction was not reduced when LS180 cells or HepG2 cells were exposed to different concentrations of paclitaxel, a microtubule destabilizer that does not interfere with nucleic acids. Taken together, these control experiments suggest that cisplatin leads to Pt-RNA, whose pro-apoptotic effects are partly mediated by TLR3. This is in line with observations showing that the knock-out or mutations of a TLR isoform decrease the efficacy of a platinum-based chemotherapy *in vivo* [27]. Because TLR3 is also known to mediate immune signaling, comprehensive gene expression profiling was performed in HepG2 cells, the cell line with pronounced responses to Pt-RNA. Pt-RNA induced the expression of nuclear factor kappa B subunit 2 by 19% ($2^{0.25}$) and interleukin-8 by 160% ($2^{1.38}$). Accordingly, this pro-inflammatory signaling pathway and the associated cytokine response appear to be activated not only by treatment with platinum drugs [28–30], but also

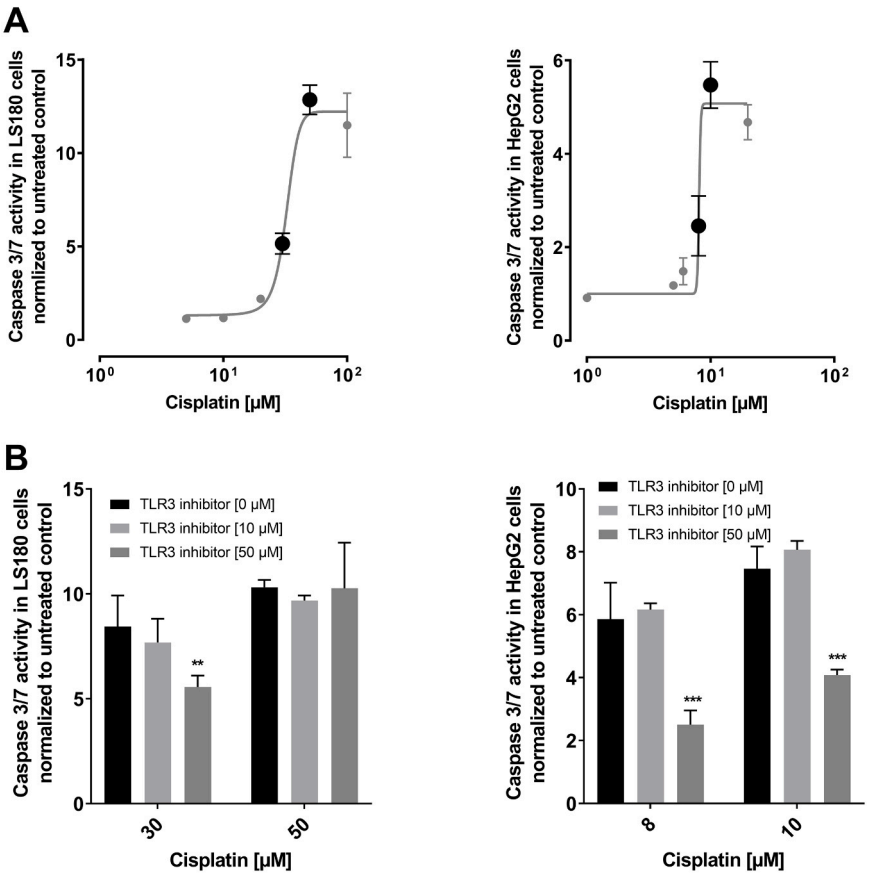

Fig. 8. Impact of TLR3 inhibition on the caspase 3/7 activity of LS180 cells (left) and HepG2 cells (right), treated with cisplatin. Cells were exposed to cisplatin. Concentrations causing sub-maximum or maximum caspase 3/7 enhancements (black circles, A) were subsequently used for evaluation of the effect of TLR3 inhibition (B). Data are shown as mean ± S.D. of triplicates. ** $P < 0.01$; *** $P < 0.001$.

by exposure to Pt-RNA. This in turn suggests that Pt-RNA is a damage-associated molecular pattern, potentially explaining the known immunomodulatory effect of (low-dose) chemotherapy with platinum drugs [31,32] and the T cell-stimulating effects of cisplatin-damaged cells [33].

This work has several limitations. First, the isolated RNA was exposed to 1 mM cisplatin, a concentration that unlikely occurs in plasma or tumor tissue after clinical administration of cisplatin [34,35]. However, the aim of this study was to assess the hypothesis of Pt-RNA-mediated apoptosis in principle. Consequently, considerable platination of RNA was strived for, which was clearly achieved. Subsequent studies should now define the extent of RNA platination that is needed for apoptosis initiation ('pharmacodynamics' of Pt-RNA-mediated apoptosis). Second, it remains open whether there is a specific (platinated) RNA sub-species that primarily mediates apoptosis. It seems conceivable that platination of certain RNA molecules alters their interaction with RNA-binding proteins, eventually influencing transcription, post-transcriptional processing, nuclear export, or decay of RNA molecules that in turn influence many fundamental biological processes [36]. For instance, platinated microRNAs could affect DNA repair, cell cycle checkpoints, and apoptosis of the host cell [37], given the potential of such microRNAs to be therapeutic targets [38]. However, selectively treating certain RNA species with cisplatin and transfecting them back into the host cell would not have reflected the biological reality when intact cells are exposed to cisplatin. Third, the models used (established cancer cell lines; *C. elegans*) may limit the general applicability of the results, especially when considering different types of cancer or organisms. However, our study tried to delineate the pro-apoptotic effects of considerably platinated RNA for the very first time. Accordingly, we focused on two cancer cell lines that have been used in the past to investigate platinum drug effects. In detail, LS180 cells [39,40] and HepG2 cells [41–43] are well-suited models for anti-cancer drug research and toxicology. Moreover, both cell lines are derived from cancer entities that can be successfully treated with platinum drugs [44,45]. To confirm the results in a multicellular organism, we used a validated apoptosis reporter *C. elegans* strain [22]. Given its selective cell division activity in the gonads and the engulfing property of respective sheath cells, cell death and subsequent formation of GFP-enriched apoptotic rings can objectively recorded and quantified in this tissue by fluorescence microscopy. Forth, we expect TLR3 to be involved in the mode of action of Pt-RNA, although other dsRNA sensors such as retinoic acid-inducible gen I (RIG-I)-like receptors or protein kinase R [46,47] may also recognize Pt-RNA and subsequently trigger downstream effects, but were not evaluated in this study. However, given the importance of TLR3 expression and activity in cancerous tissue [13–15], the relevance of this protein for Pt-RNA-mediated apoptosis was highlighted here.

In summary, the experimental data presented here document that Pt-RNA causes apoptosis and immune signaling, partly mediated by TLR3. This eventually suggests that cellular RNA could be a relevant target of platinum drugs.

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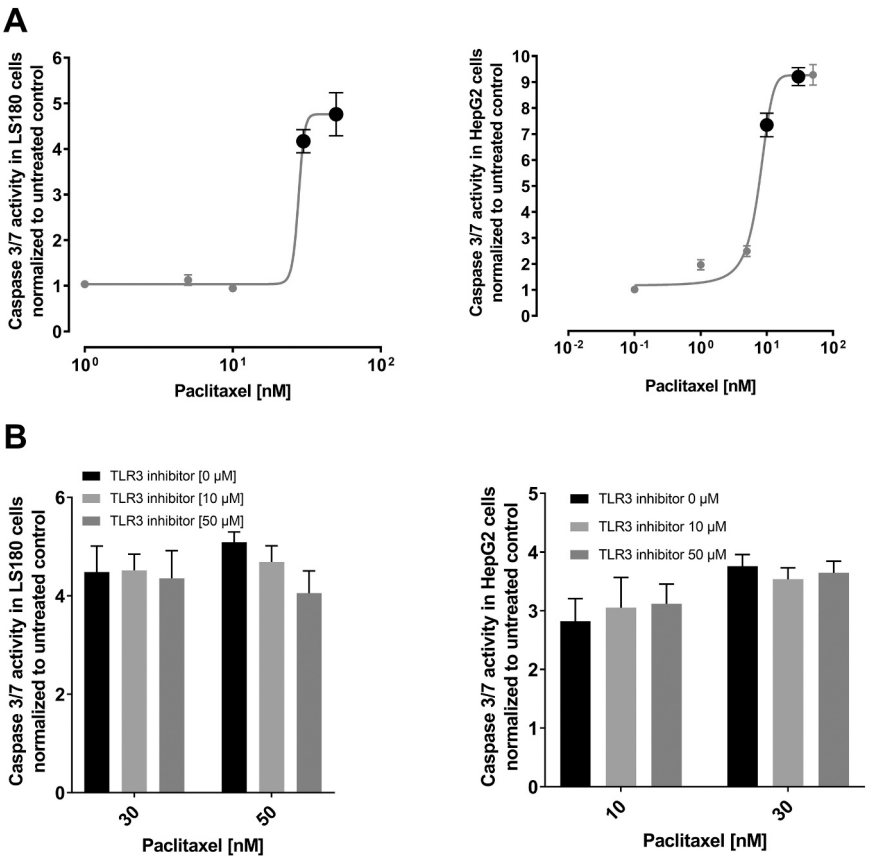

Fig. 9. Impact of TLR3 inhibition on the caspase 3/7 activity of LS180 cells (left) and HepG2 cells (right), treated with paclitaxel. Concentrations causing sub-maximum or maximum caspase 3/7 enhancements (black circles, Fig. 8A) were subsequently used for evaluation of the effect of TLR3 inhibition (Fig. 8B). Data are shown as mean ± S.D. of triplicates.

CRediT authorship contribution statement

Johanna Weiss: Writing – review & editing, Resources. **Walter E. Haefeli:** Writing – review & editing, Resources. **Dirk Theile:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Fabian Rose:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Beate Köberle:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Sebastian Honnen:** Writing – original draft, Visualization, Investigation, Formal analysis. **Cindy Bay:** Investigation, Formal analysis. **Jürgen Burhenne:** Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116450.

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