Culture media, DMSO and efflux affect the antibacterial activity of cisplatin and oxaliplatin

A. Gupta, L. Bernacchia and N.M. Kad

School of Biological Sciences, Division of Natural Sciences, University of Kent, Canterbury, UK

Significance and Impact of the Study: As well as an anticancer treatment, cisplatin possesses antibacterial activity and is active against AMR-resistant persister cells, opening the possibility of renewed use against resistant bacterial strains. Our findings provide evidence on how the composition of growth media and choice of solvent modulate the antibacterial activity of cisplatin and its analogue, oxaliplatin. These observations provide a necessary, consistent standard for assessing the antibacterial activity of platinum-based compounds, as a precursor towards their application against bacterial infection.

Introduction

Cisplatin is a platinum-based DNA crosslinker mainly used to treat a variety of cancers such as testicular, ovarian, bladder and lung (Dasari and Tchounwou 2014). It exhibits antitumor activity by forming intra- and inter-strand DNA crosslinks (Hashimoto et al. 2016). Although widely used in cancer chemotherapy, it was first discovered for its antibacterial activity against Gram-negative bacteria (Rosenberg et al. 1967) and has been shown to be effective against bacterial persister cells (Choudhury et al. 2016). The antibacterial activity was shown to be caused by the formation of DNA adducts through susceptibility studies using nucleotide excision DNA repair mutant strains (Beck et al. 1985). More recently, it has been shown that genes involved in nucleotide excision repair and SOS response in Escherichia coli are upregulated upon cisplatin treatment, providing further evidence of DNA damage in bacteria (Beaufay et al. 2020). Although a highly potent chemotherapeutic agent, cisplatin exhibits low solubility, and its activity is dependent on solvent interactions. The universal solvent, DMSO, has
Results and discussion

Cisplatin binds to DNA in both pro- and eukaryotic cells inhibiting essential processes such as transcription and replication. This DNA binding requires cisplatin to be ‘aquated’ in the cells by the replacement of a chloride ligand with water (Hall et al. 2014). Our initial studies of cisplatin’s antibacterial activity against *E. coli* MG1655 in MHBII showed no growth inhibition (MIC $>50$ mg l$^{-1}$, Fig. 1a). In contrast, in MOPS, cisplatin exhibited a MIC of 3-12 and 0-39 mg l$^{-1}$ against MG1655 and MG1655 Δ*uvrA*, respectively (Fig. 1a). We also sought to determine whether DMSO affected the inhibitory activity of cisplatin against *E. coli*. The MIC for DMSO was determined to be 10% (Fig. 1b); therefore, 2-5% was selected as the working concentration to avoid any impact on bacterial growth. Even with a low concentration of DMSO (2-5%), we found that the activity of cisplatin was reduced fourfold and eightfold against MG1655 and MG1655 Δ*uvrA*, respectively (Fig. 1a; MIC, 12-5 and 3-12 mg l$^{-1}$). A similar fourfold reduction in cisplatin activity was also observed when cisplatin was dissolved in 100% DMSO instead of 0-9% NaCl (Fig. 1c).

Previous studies have shown that sulphur-containing compounds, such as DMSO, can bind prior to aquation and impair cytotoxicity (Fischer et al. 2008; Yi and Bae 2011; Hall et al. 2014). Consistent with these previous observations, we show for the first time that DMSO also reduces cisplatin toxicity in *E. coli* and define the MIC with 2-5% DMSO clearly as 12-5 mg l$^{-1}$ (Fig. 1a).

To eliminate the efflux of cisplatin as the cause of this reduced activity, the tolC deletion from the Keio collection was transduced into MG1655 to generate the strain MG1655 Δ*tolC*. The susceptibility of this strain to cisplatin was identical to WT (MIC 3-12 mg l$^{-1}$, Fig. 1a), compared with a 64-fold difference observed for ethidium bromide inhibitory activity, against WT and Δ*tolC* (MIC, 62-5 and 0-9 mg l$^{-1}$, respectively, Fig. 1d). Ethidium bromide was used as a control since it is a known substrate for TolC that possesses DNA intercalation activity (Paixao et al. 2009). Our findings clearly demonstrate that cisplatin is not a substrate for the AcrAB-ToIC efflux pump since its MIC was not altered when we knocked out TolC, an integral part of the system.

The primary differences between MHBII and MOPS are the presence of casamino acids and beef extract, at concentrations of 17.5 and 3 g l$^{-1}$, respectively. To test if these components were responsible for the elimination of antibacterial activity in MHBII, we determined the activity of cisplatin in MOPS with the addition of either casamino acids (17.5 g l$^{-1}$) or yeast extract (3 g l$^{-1}$) (used in place of beef extract). Substitution of beef extract with yeast extract is likely to have the same impact on cisplatin activity, as reduced antibacterial activity (MIC 100 mg l$^{-1}$) has previously been observed when susceptibility testing was determined in Lysogeny broth, which comprises 5 g l$^{-1}$ yeast extract (Choudhury et al. 2016). Figure 2a shows that the antibacterial activity of cisplatin was markedly diminished upon the addition of the two media supplements. To confirm this observation is specific to cisplatin, we determined the MIC for ampicillin and nadifloxacin (bactericidal and bacteriostatic compounds, respectively), in MOPS and MOPS supplemented with either casamino acids or yeast extract. The largest change observed was approximately fourfold, well within both CLSI and EUCAST break points (EUCAST 2018; CLSI 2022) and considerably smaller than that observed with cisplatin in similar conditions (Fig. 2a). Therefore, the small increase in MIC observed in the case of ampicillin is not significant and shows that growth does not play a role in the observed cisplatin susceptibility.

To further test if the reduced activity is not dependent on growth but rather a direct interaction between cisplatin and the components of MHBII, we preincubated cisplatin in 0-9% NaCl with either casamino acids (acid hydrolysate of casein, 17.5 g l$^{-1}$) or yeast extract (3 g l$^{-1}$), for 4 and 24 h, and measured the MIC. As a control, ampicillin was subjected to the same treatment in water. An eightfold and >16-fold reduction in activity was observed when cisplatin was incubated with casamino acids (Fig. 2b). Cisplatin activity was also reduced by eightfold, upon treatment with yeast extract (Fig. 2b). Neither of the supplements influenced the activity of ampicillin (Fig. 2c), suggesting that the reduced activity of cisplatin is not linked to growth deficiency. Rather, cisplatin likely interacts with methionine and/or cysteine and is found in the acid hydrolysate of casein (casamino acids) used in MHBII, thereby reducing its availability and subsequent activity. Because acid treatment of cysteine results in numerous products (Inglis and Liu 1970), we simplified our approach by studying cisplatin in the presence of MOPS supplemented with l-cysteine (4-6 g kg$^{-1}$) and l-methionine (32 g kg$^{-1}$), which reflect the amounts estimated to be present upon acid hydrolysis of casein (Pieniazek et al. 1974). In these conditions, we observed a significant reduction in cisplatin activity (MIC, ≥25 mg l$^{-1}$, Fig. 3a). As in Fig. 2a, ampicillin exhibited a fourfold reduction in activity under the same conditions (Fig. 3b). We expect that the sulphur present in cysteine...
and methionine react with one of the cisplatin’s platinum atoms, leading to its inability to bind to DNA, akin to DMSO (Hall et al. 2014). Our results also correspond to the antagonistic role of methionine and cysteine on the antibacterial activity of cisplatin shown previously in Helicobacter pylori (Lettl et al. 2020). We suspect that the same observations will be reproduced in other Gram-negative and Gram-positive organisms, as the reduction in cisplatin’s antibacterial activity is a consequence of the choice of growth media. However, we acknowledge that the lack of bacterial diversity in the current study is a potential limitation and hope to address this in future work involving platinum-based anti-chemotherapeutic agents. However, our results still provide a platform for the accurate study of the true antibacterial activity of platinum-based compounds.

We also show that the reduction in antibacterial activity in MHBII is not specific to cisplatin. Figure 3c shows that oxaliplatin (a structural analogue of cisplatin) was less active against MG1655 (MIC 100 mg l⁻¹, Fig. 3c) compared with cisplatin. Furthermore, oxaliplatin shows no activity in MHBII (MIC >400 mg l⁻¹) and reduced activity in the presence of l-cysteine and l-methionine individually or in combination (MIC>400 mg l⁻¹, Fig. 3d).

In summary, our results explain the necessity for high cisplatin concentrations used in previous studies (Beck et al. 1985; Keller et al. 2001; Choudhury et al. 2016) and provide a framework for the accurate study of cisplatin-based bacterial cytotoxicity for future studies. We recommend the use of minimal media with limited DMSO concentrations of <2.5%. Using the conditions laid out in this study, we hope to provide a uniform standard for studying bacterial cytotoxicity with platinum-based compounds such as cisplatin and oxaliplatin.

**Material and methods**

**Bacterial strains, media culture conditions**

The strains used in this study include *E. coli* MG1655, MG1655 ΔuvrA and MG1655 ΔtolC. The two knockout strains were generated by P1 transduction of the respective gene deletions from the Keio collection (Baba et al. 2006). Luria Bertani broth and agar were used to maintain
bacterial strains (Sigma, Dorset, UK). Strains were subcultured in Mueller–Hinton Broth II (MHBII; Sigma) or MOPS (Melford, Berkshire, UK) minimal medium (pH 7.14) (Neidhardt et al. 1974) supplemented with 0.2% glucose, 1.32 mmol l⁻¹ K₂HPO₄ and 0.1 μg ml⁻¹ thiamine, for susceptibility testing. All strains were grown at 37°C with vigorous aeration.

Antimicrobials and chemicals

Cisplatin, oxaliplatin and nadifloxacin were purchased from Sigma, and ampicillin was sourced from Melford, Suffolk, UK. Media supplements—casamino acids and yeast extract were purchased from BD Scientific, Berkshire, UK.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of cisplatin, oxaliplatin, ampicillin and nadifloxacin, was determined by broth microdilution against MG1655 and MG1655 ΔuvrA, according to CLSI guidelines (CLSI 2012), with the following adjustments. After 16 h incubation of 96-well plates at 37°C, the MIC was determined by the addition of 50 μl of resazurin (0.3 mg ml⁻¹) in MOPS/tricine buffer (pH 7.8) to the plates and incubation at 37°C for a further 4 h. A colour change from blue to pink indicated growth and the MIC was defined as the lowest concentration which prevented a colour change (Palomino et al. 2002; Sarker et al. 2007). The use of resazurin provides a highly accurate determination of the MIC since even one ‘live’ cell will cause a colour change. Top stocks of cisplatin were made at 0.5 mg l⁻¹ in either 0.9% NaCl or DMSO due to its low solubility and were subsequently used as the starting concentration for susceptibility testing, yielding an unconventional doubling dilution series. Obtained MICs were not rounded off to the nearest value indexed to the base 2.
Author contributions
AG and NMK designed the study and drafted the manuscript; AG and LB conducted the experiments and collected all the data; AG analysed the data; All authors have read and approved the final manuscript.

Acknowledgements
The authors would like to thank the other members of Kad group for helpful discussions on the topic. Open access funding enabled and organized by ProjektDEAL.

Funding information
This work was supported by Cancer Research UK. grant no. – [A30456].

Conflict of Interest
None to declare.

Data Availability Statement
Data available on request from the authors

References
Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M. et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol 2, 2006–2008.
Beaufay, F., Quarles, E., Franz, A., Katamanin, O., Wholey, W.Y. and Jakob, U. (2020) Polyphosphate functions in vivo as an iron chelator and Fenton Reaction inhibitor. mBio 11, e01017-20.
Beck, D.J., Popoff, S., Sancar, A. and Rupp, W.D. (1985) Reactions of the UVRABC excision nuclease with DNA damaged by diamminedichloroplatinum(II). Nucleic Acids Res 20, 7395–7412.
Choudhury, N., Wood, T.L., Vasquez-Martinez, M., Garcia-Contreras, R. and Wood, T.K. (2016) DNA-crosslinker cisplatin eradicates bacterial persister cells. Biotechnol Bioeng 113, 1984–1192.
CLSI (2012) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically: Approved Standard. 19th edn. Wayne: Clinical and Laboratory Standards Institute.
CLSI (2022) Performance Standards for Antimicrobial Susceptibility Testing. Supplement M100. 32nd edn. Wayne: Clinical and Laboratory Standards Institute. http://em100.edaptivedocs.net/dashboard.aspx

Figure 3 Reduced antibacterial activity of platinum drugs, cisplatin and oxaliplatin in the presence of sulphur containing amino acids. (a) Cisplatin checkerboard assays with L-cysteine and L-methionine, individually and together; (b) Ampicillin checkerboard assays with L-cysteine and L-methionine, individually and together; (c) Antibacterial activity of oxaliplatin against MG1655 in MOPS, MHBII and MOPS treated with components of MHBII; (d) Oxaliplatin checkerboards with L-cysteine and L-methionine. (n ≥ 3, individual plate photographs are representative of at least 3 independent replicates)
Dasari, S. and Tchounwou, P.B. (2014) Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol 740, 364–378.

Dernell, W.S., Straw, R.C., Withrow, S.J., Powers, B.E., Fujita, S.M., Yewey, G.S., Joseph, K.F., Dunn, R.L. et al. (1997) Apparent interaction of dimethyl sulfoxide with cisplatin released from polymer delivery devices injected subcutaneously in dogs. J Drug Target 5, 391–396.

EUCAST. (2018) Breakpoint Table for Interpretation of MICs and Zone Diameters. Version 8.1. The European Committee on Antimicrobial Susceptibility Testing. https://www.eucast.org/

Fischer, S.J., Benson, L.M., Fauq, A., Nayor, S. and Windebank, A.J. (2008) Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. Neurotoxicology 29, 444–452.

Hall, M.D., Telma, K.A., Chang, K.E., Lee, T.D., Madigan, J.P., Lloyd, J.R., Goldlust, I.S., Hoeschele, J.D. et al. (2014) Say no to DMSO: Dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. Cancer Res 74, 3913–3922.

Hashimoto, S., Anai, H. and Hanada, K. (2016) Mechanisms of interstrand DNA crosslink repair and human disorders. Genes Environ 38, 9.

Inglis, A.S. and Liu, T.Y. (1970) The stability of cysteine and cystine during acid hydrolysis of proteins and peptides. J Biol Chem 245, 112–116.

Keller, K.L., Overbeck-Carrick, T.L. and Beck, D.J. (2001) Survival and induction of SOS in Escherichia coli treated with cisplatin, UV-irradiation, or mitomycin C are dependent on the function of the RecBC and RecFOR pathways of homologous recombination. Mutat Res 486, 21–29.

Lettl, C., Schindele, F., Testolin, G., Bar, A., Rehm, T., Bronstrup, M., Schobert, R., Bilitewski, U. et al. (2020) Inhibition if type IV secretion activity and growth of Helicobacter pylori by cisplatin and other platinum complexes. Front Cell Infect Microbiol 10, 602958.

Massart, C., Le Tellier, C., Gibassier, J. and Nicol, M. (1993) Modulation by dimethyl sulfoxide of the toxicity induced by cis-diaminedichloroplatinum in cultured thryocytes. Toxicol In Vitro 7, 87–94.

Neidhardt, F.C., Bloch, P.L. and Smith, D.F. (1974) Culture medium for enterobacteria. J Bacteriol 119, 736–747.

Paixao, L., Rodrigues, L., Couto, L., Martins, M., Fernandes, P., de Carvalho, C., Monteiro, G.A., Sansonetto, F. et al. (2009) Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli. J Biol Eng 3, 18.

Palomino, J.C., Martin, A., Camacho, M., Guerra, H., Swings, J. and Portaels, F. (2002) Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 46, 2720–2722.

Pieniazek, D., Rakowska, M., Szkiladziowa, W. and Grabarek, Z. (1974) Estimation of available methionine and cysteine in proteins of food products by in vivo and in vitro methods. B Jr Nutr 34, 175–190.

Rosenberg, B., Camp, L.V., Grimley, E.B. and Thomson, A.J. (1967) The inhibition of growth or cell division in Escherichia coli by different ionic species of platinum (IV) complexes. J Biol Chem 6, 1247–1352.

Sarker, S.D., Nahar, L. and Kumarasamy, Y. (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 42, 321–324.

Yi, Y.W. and Bae, I. (2011) Effects of solvents on in vitro potencies of platinum compounds. DNA Repair (Amst) 10, 1084–1085.