Impact of anticancer chemotherapy on the extension of beta-lactamase spectrum: an example with KPC-type carbapenemase activity towards ceftazidime-avibactam

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Through their action on DNA replication, anticancer chemotherapies could increase the basal mutation rate in bacteria and increase the risk of selecting antibiotic resistant mutants. We investigated the impact of several drugs on a beta-lactamase model using KPC-type carbapenemase-producing Enterobacteriaceae. We studied the impact of anticancer chemotherapies used in pediatric hematologic malignancies on 7 clinical isolates of Enterobacteriaceae producing KPC-type carbapenemases. We compared the mutation rates from cultures with/without chemotherapy on ceftazidime-avibactam, rifampicin and ceftazidime-avibactam combined with meropenem media. Mechanisms of ceftazidime-avibactam resistance were explored on a subset of mutants. After exposure to some cytotoxic molecules, the bacterial mutation rates leading to ceftazidime-avibactam and to rifampicin resistance increased up to 104-fold while we observed no emergence of resistant mutants (frequency of <10−10) on a meropenem combined with ceftazidime-avibactam media. Compared to the parental strains, an increased susceptibility to meropenem was observed in the ceftazidime-avibactam resistant mutants. The \textit{bla}_{Kpc} genes of ceftazidime-avibactam mutants harbored either mutations, deletions or insertions, especially in the region encoding the \Omega-loop of the KPC-type carbapenemase. Anticancer chemotherapy can increase the mutation rates of bacteria accelerating the extension of KPC-type carbapenemases towards ceftazidime-avibactam, one of the last resort antimicrobial chemotherapy.

Patients treated with anticancer chemotherapy drugs (ACD) are vulnerable to infectious diseases due to immunosuppression and to the direct impact of ACD on their intestinal microbiota1,2. Some anticancer drugs like mitomycin C are known to have a bacteriostatic or bactericidal effect3,4 and can also be mutagenic on bacteria5. It is suggested that the dysbiosis can participate to intestinal mucositis5 that enhances the risk of bacterial translocation to the blood, requiring an antibiotic therapy, which may in turn favor the emergence of antibiotic resistant bacteria. The growing carriage of Carbapenem-Resistant Enterobacteriaceae (CRE) increases the risk of invasive infections with these resistant bacteria responsible for a mortality rate over 30%6. \textit{Klebsiella pneumoniae} carbapenemase (KPC) was described for the first time in 1996. It is now a common carbapenem resistance mechanism among Enterobacteriaceae in the USA, Israel, Asia, Latin America, and South Europe, reaching a prevalence rate of 66.5% among \textit{K. pneumoniae} isolated in Greece10,11. Carbapenem-resistant \textit{K. pneumoniae} bacteremia are responsible for 73% of 30-day mortality in cancer patients12. The recent combination ceftazidime-avibactam (CZA) has been approved in 2015 in the USA and in 2016 in Europe13,14. CZA demonstrates excellent \textit{in vitro} activity against CRE of KPC-type, and is associated with a decreased mortality rate in treated patient15,16. Its
efficacy, broad antibacterial spectrum and safety, led to an increase in its use, notably for immunosuppressed patients treated with ACD. Unfortunately, some mutations in \( \text{bla}_{\text{KPC}} \) or in genes encoding porins lead to resistance to CZA, thus limiting the benefit of this last resort strategy. Moreover, several ACD interfere with DNA replication and increase the mutation rate of eukaryotic cells. It has been showed in vitro that anticancer chemotherapy drugs could enhance the emergence of antibiotic-resistant pathogenic bacteria, mostly through activation of the SOS response. The alkylating agent dacarbazine, the topoisomerase inhibitors epirubicin and daunorubicin, and the pyrimidine analogue azacitidine were shown to accelerate the bacterial evolution. We thus hypothesized that ACD treatment could accelerate the modification of beta-lactamase spectrum and thus the emergence of CZA-resistant mutant in KPC-producing Enterobacteriaceae.

In this study, we investigated the impact of several ACD on beta-lactamase evolution. We chose to evaluate the emergence of CZA-resistant mutants in KPC-producing Enterobacteriaceae. We subsequently evaluated an antimicrobial combination to limit the emergence of resistant mutants to this last resort antibiotic. Last, we sequenced and cloned the \( \text{bla}_{\text{KPC}} \) genes of the CZA-resistant mutants to identify the mutations accounting for the enhanced resistance to CZA.

**Results**

**Mutation rate.** The culture of *Enterobacter cloacae* KPC-3 (RD26) and *E. coli* KPC-2 (RD29) with ACD modified the frequency of resistant mutants to CZA and rifampicin in a variable manner depending on the isolates and the cytotoxic molecules. Resistance to rifampicin is a classical model used to evaluate mutation rates. Control conditions without chemotherapy molecule showed that the strains have a normal mutational rate against rifampicin. Compared to the control culture without ACD, the frequency of emergence of rifampicin-resistant mutants and of CZA-resistant mutants increased up to \( 10^4 \) and to \( 10^3 \) after exposure to some cytotoxic molecules (Fig. 1 and Supplementary Table S1).
In details, the frequency of emergence of CZA-resistant mutants from *E. cloacae* RD26 significantly increased after incubation with azacitidine (p = 0.0006), dacarbazine (p = 0.003), and mitoxantrone (p = 0.008). Azacitidine had the highest impact on mutation rates with an increase up to 10^{-4}-fold. Dacarbazine (p = 0.002) and mitoxantrone (p = 0.04) significantly increased the frequency of CZA-resistant mutants from *E. coli* KPC-2 (isolate RD29), up to 10^{-3}-fold increase for dacarbazine (Supplementary Table S2).

Dacarbazine increased the rate of rifampicin-resistant mutants (p = 0.005), with a maximum of 10^{-4}-fold increase (Supplementary Tables S1 and S2) for *E. coli* RD29. Azacitidine also had a significant impact for both strains (p = 0.001 for *E. cloacae* RD26, and p = 0.003 for *E. coli* RD29) (Supplementary Tables S1 and S2) while mitoxantrone, cyclophosphamide, daunorubicin, mercaptopurine and cytarabine had no significant effect.

Concerning meropenem, whatever the ACD used with the RD26 isolate, no mutation rate could be determined since confluent growth was observed at the concentration used. However, no resistant mutant was observed on the medium with CZA combined with meropenem and therefore the mutation rate is < 10^{-10} (Supplementary Tables S1 and S2).

**Confirmation of the ACD impact on the mutation rate with azacitidine as an example, on 5 other strains.** In order to evaluate the capability of ACD, and especially azacitidine which is one of the most used molecules in pediatric hematologic malignancies, to increase mutation rate of different species or isolates, five non-related clinical isolates of different sequence types of *K. pneumoniae* (Supplementary Tables S3 and S4) were tested against azacitidine 0.5 mg/L. Similar results with increased frequencies of rifampicin- and CZA-resistant mutants were observed but no resistant mutant on the medium combining meropenem and CZA.

**Confirmation of the ACD impact on the MIC increase by comparing parental isolates with transformants.** The MICs of CZA on nine CZA-resistant mutants derivatives of RD26 selected randomly were 4- to 16-times higher than that of their parental isolate. This resulted in some MICs incompatible with therapeutic use. In contrast, the meropenem MIC decreased subsequently, from 16 mg/L with the parental isolate to 0.094–2 mg/L in the CZA-resistant mutants. The MIC of meropenem combined with CZA stayed very low (< 0.064 mg/L). MICs of CZA, meropenem, and meropenem/CZA performed on *E. coli* Top 10 carrying the *bla*KPC genes borne in the plasmid pBR322 gave similar results. A similar level of *bla*KPC mRNA transcription was observed for the recombinant mutants compared to their recombinant parental strain (Supplementary Table 5) suggesting similar KPC expression. Resistance to CZA was therefore very likely to be due to *bla*KPC gene alteration (Table 1). To explore the *bla*KPC gene mutation, we sequenced it in these nine RD26 CZA-resistant mutants as well as in seven transformants of *E. coli*. These revealed either point mutations, deletions, or insertions in the region of the omega-loop of the KPC (Table 1). Indeed, two mutants carried the mutation R164P, two mutants were mutated in position 179 (D179H and D179Y), and one mutant carried the mutation T243M. We also observed mutations leading to the insertion of 3 amino acids (SAIPG) between G175 and D176, of three amino acids mutated in position 179 (D179H and D179Y), and one mutant carried the mutation T243M. We also observed the selection of resistant mutants in patients15,17. Reports on the emergence of CZA-resistant CRE soon after its launch led to the publication of a worrying report on rapid risk assessment in June 2018 by the European Centre for Disease prevention and Control26.

**Discussion** We showed here that certain ACD can stimulate the bacterial mutagenesis and can lead to mutations in *bla*KPC. Some of these mutations were previously shown to increase the catalytic efficiency of KPC towards ceftazidime resulting in the failure of ceftazidime-avibactam22,23.

Anticancer drugs can have genotoxic effects24 and thus stress the bacteria, through their impact on DNA and cell division. Stress activates the SOS response that increases the mutation rates of bacteria19,21,25. CZA may offer a significant advantage over antimicrobials agents which may keep *in vitro* activity against CRE, such as colistin, fosfomycin, and tigecycline but which are limited by concerns over efficiency and/or toxicity16. Although CZA is considered as one of the last resort treatment in case of infection with KPC-producing strains8 its use may lead to the selection of resistant mutants in patients15,17. Reports on the emergence of CZA-resistant CRE soon after its launch led to the publication of a worrying report on rapid risk assessment in June 2018 by the European Centre for Disease prevention and Control26.

In this study, we observed that when bacteria are incubated with certain ACD such as azacitidine or dacarbazine, the increase frequency of resistant mutants (up to 10^{-6}) and their high MIC to CZA are particularly worrying as it could lead to the treatment’s failure in case of severe infection.

As *E. cloacae* RD26 was not susceptible to meropenem, CZA would have been an option for the treatment in case of infection. Hence, we observed confluent growth on the medium containing meropenem at therapeutic concentration. A valuable result to point out is the absence of selection of cross-resistant mutants to the association CZA-meropenem. Indeed, whatever the mutation rate after a culture with ACD and selection on antibiotic medium, we found no emergence of resistance to this association at threshold detection of 10^{-8}. This is of importance as it widens the therapeutic possibilities to reduce the risk of selection of resistant mutants. Avibactam protects meropenem from hydrolysis by the KPC β-lactamase and restores the ceftazidime activity in the case of production of ESBL or AmpC β-lactamases. Furthermore, a tradeoff between carbapenemase activity and resistance to CZA has already been described after *in vivo* and *in vitro* selection3,27,30. These include mutations in position 164 and 179 which are located in the Ω-loop of this class A β-lactamase. The Ω-loop (residues 164–179) is a hot spot for substitutions that extend the substrate spectrum of many class A enzymes. It contains Glu164 and Asn170.
two essential residues required for priming a water molecule for deacylation of the β-lactam. Mutations in the ƞ-loop can modify salt bridge (in particular between Arg164 and Asp179) and thus enhances the flexibility of the loop allowing a better binding and hydrolysis of ceftazidime. Similarly, insertions or deletions that occur in the ƞ-loop modify its structure and steric bulk22,23,29 and thus modify the access of ceftazidime. The effect of methionine for threonine substitution at position 243 is unclear27 as well as deletions of 2 amino acids (GV) between positions 234 to 242, these mutations could increase flexibility of the 3-strand and allow the aminothiazole ring of ceftazidime to sink deeper into the active site upon binding. Finally, the effect of the insertion of 3 amino-acid (DDK) between positions 273 and 274 is not known, but its spatial proximity to the active site is probably significant31.

Given the high mutation rates and potential clinical implications of therapeutic failure, it seems therefore reasonable to consider an association such as CZA with meropenem in the event of treatment of a severe infection by a KPC-producing Enterobacteriaceae in a patient treated with ACD and especially with dacarbazine, azacitidine, and mitoxantrone.

One of the biggest difficulties in carrying out this study was to choose the concentrations of chemotherapy molecules. Indeed, there are few data on therapeutic concentrations in pediatrics and to our knowledge none on concentrations obtained in stool. We therefore chose concentrations compatible with the blood concentrations19,32,33. We hypothesized that the diffusion of ACD in the intestinal compartment could increase the diversity of intestinal microbial population by modifying the basal mutation rate. Resistant mutants can then be selected after exposure to antibiotic treatment, even not administered concomitantly with the ACD treatment. This remains to be evaluated in vivo.

In conclusion, using two KPC-producing Enterobacteriaceae strains as a model, we found that in vitro incubation with certain ACD (i.e. azacitidine, dacarbazine, mitoxantrone) enhance the bacterial mutagenesis and the subsequent emergence of antibiotic-resistant mutants. We confirmed the capability of azacitidine to generate high rates of mutants on five different sequence types of KPC-producing K. pneumoniae., selected after exposure to cytotoxic agents. Transformants were obtained by cloning bla_KPC (wild-type and mutated) in pBR322 plasmids and electroporated in E. coli Top10.
resistance. This observation challenges the therapeutic management of patients when dealing with KPC-type carbapenemase producing Enterobacteriaceae.

Methods

**Bacterial isolates.** In a first part, we studied two multiresistant Enterobacteriaceae producing KPC-type carbapenemases: a clinical isolate of Enterobacter cloacae harboring bla\(_{\text{KPC-2}}\) (RD26) and of Escherichia coli harboring bla\(_{\text{KPC-2}}\) (RD29). MICs determined by the E-test\(^\circledR\) method (bioMérieux, France) on Mueller Hinton agar (MHA) were of 16, and 0.25 mg/L for meropenem, and 4 and 0.5 mg/L for CZA for RD26 and RD29, respectively.

In a second part, we studied five multiresistant KPC-producing *K. pneumoniae* clinical isolates: RD27 (bla\(_{\text{KPC-2}}\), DD34 (bla\(_{\text{KPC-3}}\), DD36 (bla\(_{\text{KPC-3}}\), DD37 (bla\(_{\text{KPC-2}}\), and DD38 (bla\(_{\text{KPC-2}}\) whose MIC to meropenem were of >32, 6, 16, 6, and 1 mg/L and to CZA of 8, 4, 2, 1, and 4 mg/L respectively.

**Genome sequencing and analysis.** The DNA of all isolates was extracted using the MOBIO kit (Qiagen) after an overnight growth in Luria-Bertani (LB) broth. The Nextera XT kit (Illumina, San Diego, California, USA) was used to prepare libraries. Sequencing was performed on a MiniSeq \(2 \times 150\) cycles (Illumina technology). The SPAdes assembler was used to construct assemblies. Contigs <500 bp were removed. We performed multilocus sequence typing (MLST)\(^{34}\), identification of acquired resistance genes by using ResFinder 3.0\(^{35}\) and SerotypeFinder 1.1.\(^{36}\)

The quality of the sequencing data was estimated using standard metrics. Mean M50 was of 266,195 bp and mean coverage was of 84. Quality sequencing data are summarized in Supplementary Table S4. Raw sequences of the 7 isolates were deposited in GenBank under BioProject number: PRJNA542787. Whole genome sequencing was performed on 15 clinical isolates: RD27, RD29, RD26, DD34, DD36, DD37, DD38, 5 *E. coli* and 5 *K. pneumoniae*, producing KPC-type carbapenemases: a clinical isolate of *Enterobacter cloacae* of ST131 and serotype O16 and had both bla\(_{\text{KPC-2}}\) and bla\(_{\text{TEM-1}}\). *E. cloacae* had the bla\(_{\text{KPC-3}}\), bla\(_{\text{OXA-4}}\), and bla\(_{\text{TEM-1}}\) and the 5 *K. pneumoniae* were respectively of ST-258, ST-11, ST-101, ST-307 and ST-312 and produced either bla\(_{\text{KPC-2}}\) or bla\(_{\text{KPC-3}}\) (Supplementary Table S4).

**Determination of mutant frequencies.** Mid-log cultures of either *E. coli* RD29 or *E. cloacae* RD26 in MH broth was diluted to \(10^{-4}\) and cultured overnight with and without ACD. We evaluated seven ACD used in pediatric hematologic malignancies at concentrations close to the plasmatic concentrations\(^{37}\). The pyrimidine analog cytarabine was used at 0.5 mg/L\(^{37}\). The two antimetabolites azacitidine and mercaptopurine were used at 0.5 and 30 mg/L, respectively\(^{38,39}\). The two alkylating drugs cyclophosphamide and dacarbazine were used at 2.5 and 10 mg/L\(^{40-42}\). The topoisomerase inhibitor daunorubicin was used at 50 mg/L\(^{37}\). The anthracycline mitoxantrone was used at 5 mg/L\(^{43}\).

We evaluated the basal mutation rate of the tested bacteria by plating appropriate dilutions of overnight cultures on MHA and MHA containing rifampicin (RMP) at 50 mg/L. The frequency of emergence of antibiotic-resistant mutants was calculated\(^{40,41}\) by plating overnight cultures on MHA and MHA containing 4 MIC of CZA (4:1 ratio) or meropenem (20 mg/L). We also evaluated the frequency of emergence of *E. cloacae* resistant to the combination CZA (4 MIC) – meropenem (MEM) 20 mg/L. This concentration of meropenem is compatible with the serum concentration obtained by continuous infusion of meropenem and above the MIC of the isolates\(^{44}\). All tests were done four times independently. In order to confirm and extend our results, five KPC-producing clinical isolates of *K. pneumoniae* of different ST were submitted to azacitidine on five independent experiments. The frequency of resistant mutants was then calculated as the number of resistant colonies divided by the number of plated cells. The results are expressed as the ratios of the mutant frequency with ACD on mutant frequency without ACD.

**MIC determination.** MICs against CZA, MEM and MEM in combination with CZA were assessed with the E-test\(^\circledR\) method (bioMérieux, France) according the manufacturer’s recommendations. To determine the MIC of MEM combined to CZA, one CZA strip was placed on MHA for 30 min, then removed, and a MEM strip was placed on top of the gradient of the CZA\(^{45}\). The value at which the inhibition zone intersected the scale on the MEM E-test strip was considered as the MIC of MEM combined to CZA.

**Identification of mutations involved in the resistance to CZA.** To identify the mutations leading to the resistance to CZA, bla\(_{\text{KPC}}\) of nine randomly selected mutants was PCR amplified using a high-fidelity polymerase (Pfu DNA polymerase) and sequenced with the primers KPC-F (5′-TCACTTATCGGCCGTCATGTTTT-3′) and KPC-R (5′-ATCCCTGAGGCCGGAATCTCA-3′). (We did not search for mutations in the other bla genes that were present, nor in PBPs (likely PBP3) that may also contribute to our results).

**Construction of plasmids expressing bla\(_{\text{KPC-3}}\) and derivatives.** pBR322 plasmid was used as template for PCR amplification of the whole plasmid except bla\(_{\text{TEM}}\) using specific primers that also contained overlapping regions of 5′ and 3′ regions of bla\(_{\text{KPC}}\) (for subsequent Gibson Assembly) of RD26 isolate. bla\(_{\text{KPC}}\) was PCR amplified from the DNA extracts of RD26 and some of its CZA-resistant derivatives (RD26-2, -3, -5, -6, -7, -8, and -9) with specific primers that also contained overlapping regions of 5′ and 3′ regions of pBR322 plasmid. PCR products were purified with a 1-h digestion with DpnI enzyme to remove the matrix DNA and by a gel purification (QIAquick Gel Extraction Kit, Qiagen). Resulting DNA was quantified using Qubit™ dsDNA HS Assay Kit (Thermofisher). Gibson reaction was carried out using a molar ratio of 1:1 backbone: insert in a total of 20 μl reaction mix and incubated at 50 °C for 1 hour.

The total volume of Gibson reaction was dialysed against distilled water for 30 min. One μl was then electroporated in 15 μl of One Shot™ TOP10 Electrocomp™ E. coli (Invitrogen) and then incubated in 500 μl of LB broth for 1 hour at 37 °C with shaking at 250 rpm. The transformants were plated on LB agar plates containing 10 mg/l tetra-cycline and further incubated overnight at 37 °C. Transformants obtained were PCR amplified using a high-fidelity
Pfu DNA polymerase and sequenced with the primers pbr322-KPC-F GTCTGACATTGGCAGCCGTGTA and pbr322-KPC-R AGGAGAGTATGTCAGTCTGAGCC.

**Quantitative reverse transcription-PCR (RT-PCR).** RNA from a Mueller Hinton broth culture was extracted with the Direct-zol RNA kit followed by a DNase treatment (zymoresearch, Irvine, USA) specified by the manufacturer.

Transcriptome analysis was performed using 10 ng total RNA on 3 genes: *bla*KPC, *dinB* a previously described housekeeping gene used for normalization and *tetA*, a *pBR322* plasmidic gene coding for tetracyclin resistance also used for normalization. We used the following primers: KPC-(C-GGGGAACATTGCATTAAC, R-AGGGCTTGGAATGAGCTGCAC), *dinB*-(F-GGCCGATATCCCTATTGCTA, R-AGGCCTCTTTGTAAGGCTCA) and *tetA*-(F-GTGCAGGATTGGCATAAAGGGAG, R-TGCGCGAAAATGACCCAGAG). The KAPA SYBR® FAST One-Step kit was used for PCR. Reverse transcription and amplification were performed with an LC480 Light Cycler (Roche®) in one step with the following cycling parameters: 30 min at 42 °C for reverse transcription, 3 minutes at 95 °C for reverse transcriptase inactivation and Taq activation followed by 45 cycles of 10 seconds at 95 °C, 20 seconds at 64 °C and 1 second at 72 °C. Assays were performed in triplicates. The cycle threshold (Ct) was automatically determined by using the Second Derivative Maximum Method included in the LC480 software. For each isolate, a mean Ct was calculated using the 3 replicates. The fold change in gene expression comparing mutants and pBR322-RD26 was calculated by using the 2−ΔΔCt method.

**Statistical analysis.** The mutants frequencies were analyzed with the Fluctuation AnaLysis CalculatOR (FALCOR) program. We used the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method; validated in low replication models; followed by a Student's t-test to conduct the statistical analysis. The chosen significance threshold was 0.05 for all tests.

**Accession numbers.** Whole genome sequencing data were submitted to NCBI under SRA accession: PRJN542787. *bla*KPC genes were submitted to GenBank under accession numbers: RD26-3 MN091856; RD26-6 MN091857; RD26-7 MN091858; RD26-9 MN091859.

**Data availability**

All data generated or analysed during this study are included in this published article (and its Supplementary Information Files).

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Author contributions
C.A.H. and A.Bi. performed all experiments. S.B., D.H., A.Ba., M.E., T.S., R.T., C.D., O.T. made critical revisions and arguments for the paper. C.A.H., S.B., D.H., O.T. and A.Bi. analyzed the results and wrote the manuscript. All authors reviewed and approved the final manuscript.

Competing interests
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