Inhibition of Fosfomycin Resistance Protein FosA by Phosphonoformate (Foscarnet) in Multidrug-Resistant Gram-Negative Pathogens

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ABSTRACT FosA proteins confer fosfomycin resistance to Gram-negative pathogens via glutathione-mediated modification of the antibiotic. In this study, we assessed whether inhibition of FosA by sodium phosphonoformate (PPF) (foscarnet), a clinically approved antiviral agent, would reverse fosfomycin resistance in representative Gram-negative pathogens. The inhibitory activity of PPF against purified recombinant FosA from *Escherichia coli* (FosA³), *Klebsiella pneumoniae* (FosA^{KP}), *Enterobacter cloacae* (FosA^{EC}), and *Pseudomonas aeruginosa* (FosA^{PA}) was determined by steady-state kinetic measurements. The antibacterial activity of PPF against FosA in clinical strains of these species was evaluated by susceptibility testing and time-kill assays. PPF increased the Michaelis constant (K_m) for fosfomycin in a dose-dependent manner, without affecting the maximum rate (V_{max}) of the reaction, for all four FosA enzymes tested, indicating a competitive mechanism of inhibition. Inhibitory constant (K_i) values were 22.6, 35.8, 24.4, and 56.3 μ M for FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA³, respectively. Addition of clinically achievable concentrations of PPF ($\sim$ 667 μ M) reduced the fosfomycin MICs by $\geq$ 4-fold among 52% of the *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* clinical strains tested and led to a bacteriostatic or bactericidal effect in time-kill assays among representative strains. PPF inhibits FosA activity across Gram-negative species and can potentiate fosfomycin activity against the majority of strains with chromosomally encoded *fosA*. These data suggest that PPF may be repurposed as an adjuvant for fosfomycin to treat infections caused by some FosA-producing, multidrug-resistant, Gram-negative pathogens.

KEYWORDS fosfomycin, Gram negative, glutathione S-transferase, phosphonoformate

Fosfomycin is a phosphoenolpyruvate analogue that is produced by *Streptomyces* spp. and was first reported in 1969 (1). It exhibits antibacterial activity through inhibition of the enzyme UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase (MurA), an enzyme that catalyzes the first cytoplasmic step of bacterial cell wall biosynthesis. Fosfomycin has bactericidal activity against a wide range of Gram-positive and Gram-negative bacteria and has become one of the first-line agents recommended for the treatment of uncomplicated urinary tract infections, in light of rising rates of resistance to more commonly used agents, including fluoroquinolones and trimethoprim-sulfamethoxazole, in *Escherichia coli* (2).

In Gram-negative bacteria, one of the most prevalent mechanisms of resistance to fosfomycin is production of the metalloenzyme FosA (3). FosA is a Mn²⁺- and K⁺-dependent dimeric glutathione S-transferase (GST) that catalyzes the addition of

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TABLE 1 Steady-state kinetic parameters and PPF inhibition constants for different FosA enzymes

Enzyme	Fosfomycin ^a			Phosphonoformate ^b	
	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	IC ₅₀ (μM)	K_i (μM)
FosA ^{KP}	12.8 ± 1.0	8,854 ± 215	691.7	18.2 ± 1.58	22.6 ± 2.6
FosA ^{EC}	23.6 ± 6.0	11,129 ± 1,016	471.6	33.0 ± 1.12	35.8 ± 5.1
FosA ^{PA}	5.8 ± 1.4	1,875 ± 110	323.3	35.2 ± 1.91	24.4 ± 6.0
FosA3	15.6 ± 3.4	8,163 ± 579	523.3	139.0 ± 1.26	56.3 ± 8.3

^aMeasured with 30 mM glutathione.^bMeasured with 30 mM glutathione and 30 mM fosfomycin.

glutathione to fosfomycin, rendering the antibiotic inactive (3). FosA can be encoded on either plasmids or chromosomes. While there is significant variation in FosA amino acid sequences across species, the active site residues are highly conserved. The most prevalent plasmid-mediated *fosA* gene is *fosA3*, which is distributed in multidrug-resistant (MDR) strains of the family *Enterobacteriaceae* in East Asia and elsewhere (4–6). Other plasmid-mediated *fosA* alleles, such as *fosA5* and *fosA6*, whose origin appears to be the chromosome of *Klebsiella pneumoniae*, have also been reported (7, 8). The *fosA* gene is also widely distributed on the chromosomes of many Gram-negative species, including *K. pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (9). *E. coli* notably lacks chromosomal *fosA* and is significantly more susceptible to fosfomycin than are the aforementioned *fosA*-harboring species (10, 11).

Sodium phosphonoformate (PPF) is a pyrophosphate analogue that inhibits DNA polymerases and is used clinically for the treatment of cytomegalovirus (CMV) retinitis and refractory mucocutaneous herpes simplex virus (HSV) infections (12). Interestingly, PPF was shown previously to bind to the active site of a plasmid-encoded FosA from *S. marcescens* (FosA^{Tn2921}), where it acts as a minimal transition state inhibitor of the enzyme (13). PPF at a high concentration (1 mg/disk) can also be used in a diagnostic test to detect *E. coli* carrying plasmid-encoded *fosA*, such as *fosA3* (14). However, PPF has never been rigorously assessed for its ability to reverse fosfomycin resistance in Gram-negative pathogens that harbor *fosA*, particularly at clinically achievable concentrations of the drug. When PPF is administered intravenously as an antiviral agent (either 60 mg/kg three times daily or 90 mg/kg twice daily), the peak plasma concentrations range from 495 to 605 μM (15). Accordingly, in this study we determined the inhibitory activity of PPF in combination with fosfomycin against representative Gram-negative pathogens.

RESULTS

Sequence of FosA. The sequences of FosA in *K. pneumoniae* I1, *E. cloacae* YDC666, *P. aeruginosa* 75A7, and *E. coli* YD472 were determined by whole-genome sequencing using an Illumina NextSeq system. The amino acid sequences of FosA in these four strains shared 100%, 98%, 99%, and 100% identity with FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3, respectively, which were generated from *K. pneumoniae* HS11286, *E. cloacae* ECN1H5, *P. aeruginosa* PAO1, and *E. coli* p06607, respectively (see Fig. S1 in the supplemental material).

FosA inhibition by PPF. We first evaluated the steady-state kinetic parameters of the Michaelis constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) for recombinant purified FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3 (Table 1). In general, the k_{cat}/K_m values were similar for all four enzymes, although the K_m and k_{cat} values for FosA^{PA} differed from those determined for FosA^{KP}, FosA^{EC}, and FosA3. For all four enzymes, we found that PPF increased the K_m for fosfomycin in a dose-dependent manner but had no effect on the maximum rate of the reaction (i.e., V_{max}) (Fig. 1; also see Table S1). This pattern of inhibition is consistent with a competitive mechanism of action. The inhibitory constant (K_i) values derived from this study were calculated to be 22.6, 35.8, 24.4, and 56.3 μM for FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3, respectively

FIG 1 PPF effects on the K_m (a) and V_{max} (b) values for fosfomycin binding and catalysis by FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3. PPF increases the K_m values but not the V_{max} values. Data are plotted as the mean $\pm$ standard error from at least three independent experiments.

(Table 1). The concentrations of PPF that resulted in 50% inhibition (IC_{50}) when 30 mM fosfomycin and 30 mM glutathione were used as the substrates were similar to the K_i values and were calculated to be 18.2 ± 1.58 , 33.0 ± 1.12 , 35.2 ± 1.91 , and 139.0 ± 1.26 μ M for FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3, respectively (Table 1).

Effects of PPF on fosfomycin MICs. The fosfomycin MICs with various concentrations of PPF for an expanded panel of *E. coli*, *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* strains are shown in Table 2 and Table S2. Addition of 667 μ M PPF decreased the fosfomycin MICs by ≥ 4 -fold for 52% (29/61 strains) of the *E. coli*, *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* clinical strains tested. Addition of PPF at therapeutically achievable concentrations (167 to 667 μ M) reduced the fosfomycin MICs for *K. pneumoniae* I1, *E. cloacae* YDC666, and *P. aeruginosa* 75A7 by 4- to 8-fold but had no impact on that for *E. coli* YD472. Of note, increasing the PPF concentration further (1 and 5 mM) did not reduce the fosfomycin MIC for *E. coli* YD472 (Table 2).

TABLE 2 Fosfomycin susceptibility of clinical Gram-negative strains and *E. coli* TOP10 transformants harboring *fosA*

Strain	Fosfomycin MIC (μ g/ml) ^a					
	No PPF	167 μ M PPF	333 μ M PPF	667 μ M PPF	1 mM PPF	5 mM PPF
Clinical strains						
<i>K. pneumoniae</i> I1	256	64	32	32	32	32
<i>E. cloacae</i> YDC666	256	64	32	32	8	16
<i>P. aeruginosa</i> 75A7	128	64	64	32	32	16
<i>E. coli</i> YD472	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
<i>E. coli</i> ATCC 25922	0.5	1	2	1	1	0.5
Transformants						
<i>E. coli</i> TOP10(pFosA ^{KP})	>1,024	>1,024	>1,024	1,024	64	64
<i>E. coli</i> TOP10(pFosA ^{EC})	>1,024	>1,024	1,024	1,024	64	64
<i>E. coli</i> TOP10(pFosA ^{PA})	64	8	4	4	2	1
<i>E. coli</i> TOP10(pFosA3)	32	8	4	4	4	1
<i>E. coli</i> TOP10	1	2	1	1	2	2

^aMICs were determined by the agar dilution method, with 25 μ g/ml glucose-6-phosphate supplementation.

FIG 2 Time-kill assays of fosfomycin (Fos) with PPF against *K. pneumoniae* (a), *E. cloacae* (b), *P. aeruginosa* (c), and *E. coli* (d). The concentration of PPF was 667 μ M.

E. coli TOP10 transformants producing FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3 were also evaluated. Those producing FosA^{KP} and FosA^{EC} were highly resistant to fosfomycin, with MIC values of >1,024 μ g/ml (Table 2). In contrast, the MIC values for those producing FosA^{PA} (MIC, 64 μ g/ml) and FosA3 (MIC, 32 μ g/ml) were lower and would be considered to indicate susceptibility, according to the Clinical and Laboratory Standards Institute (CLSI) breakpoint (16). However, they still represented 32- to 64-fold increases from the baseline fosfomycin MIC of 1 μ g/ml in *E. coli* TOP10. It was notable that there were large differences in the MIC values for FosA^{KP} and FosA^{EC}, compared to FosA^{PA} and FosA3, given that these enzymes exhibited similar activity *in vitro* (Table 1). This finding may be due to a greater level of expression of FosA^{KP}, compared with FosA^{PA} (17). PPF at clinically relevant concentrations (up to 667 μ M) reduced the fosfomycin MIC values for the transformants producing FosA^{PA} and FosA3 by 16- and 8-fold, respectively, but had no measurable impact on the MIC values for those producing FosA^{KP} and FosA^{EC}; however, the MIC values for both transformants were reduced when the PPF concentration was increased to 1 or 5 mM (Table 2).

Time-kill assays. *E. coli* YD472, *K. pneumoniae* I1, *E. cloacae* YDC666, and *P. aeruginosa* 75A7 were evaluated at three concentrations of fosfomycin, with and without 667 μ M PPF (Fig. 2). PPF alone exhibited no activity with any of the strains tested. For *K. pneumoniae* I1, 256 and 512 μ g/ml fosfomycin alone exhibited bactericidal effects at 8 and 24 h, although regrowth occurred at 24 h with 128 μ g/ml

fosfomycin (Fig. 2a). The fosfomycin-PPF combination showed a bactericidal effect at all concentrations of fosfomycin tested, including 128 $\mu\text{g/ml}$. For *E. cloacae* YDC666, regrowth at 24 h was observed for all concentrations of fosfomycin (Fig. 2b). However, addition of PPF resulted in total killing at 24 h for all fosfomycin concentrations. For *P. aeruginosa* 75A7, regrowth was noted after 8 h when fosfomycin was used alone, while addition of PPF showed a bacteriostatic effect with 256 $\mu\text{g/ml}$ fosfomycin at 24 h (Fig. 2c). In contrast, for *E. coli* YD472, inhibitory effects were absent for all of the fosfomycin concentrations tested, irrespective of the concentrations of PPF (Fig. 2d).

Sugar phosphate transporters of *E. coli* YD472. *E. coli* YD472 did not have amino acid mutations in the *murA*, *glpT*, *uhpT*, *uhpA*, *uhpB*, *ptsI*, and *cyaA* genes but contained one amino acid substitution (P399S) in the *uhpC* gene. *E. coli* YD472 did not grow on M9 minimal medium with glucose-6-phosphate but grew on medium with glycerol-3-phosphate. Therefore, *E. coli* YD472 likely lacks functional UhpT, which, in combination with FosA3 production, may be responsible for the high fosfomycin MIC.

DISCUSSION

The spread of MDR bacteria has become a critical public health issue (18). Fosfomycin has broad-spectrum activity against both Gram-positive and Gram-negative bacteria, and its use for the treatment of MDR infections is an active area of investigation. For example, it has been proposed as part of combination regimens for the treatment of KPC-producing *K. pneumoniae* infections (19). However, emerging resistance to fosfomycin is increasingly recognized as a concern (20, 21). While plasmid-mediated *fosA* genes such as *fosA3* can confer fosfomycin resistance in *E. coli*, which as a species lacks *fosA* (4–6), many other Gram-negative species intrinsically carry *fosA* on the chromosome (9). As we recently reported in the identification of plasmid-mediated *fosA6* in *E. coli* originating from the *K. pneumoniae* chromosome (8), chromosomal *fosA* confers reduced susceptibility or resistance to fosfomycin. In this respect, FosA inhibitors have the potential to restore or to potentiate the activity of fosfomycin against Gram-negative bacteria that produce FosA.

Our data show that PPF is a competitive inhibitor with respect to fosfomycin binding to FosA from *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa*, as well as FosA3 in *E. coli*. PPF showed similar inhibitory potencies against FosA^{KP}, FosA^{EC}, and FosA^{PA}, whereas its activity against FosA3 was somewhat decreased (Table 1). Importantly, PPF at therapeutically achievable concentrations reduced the fosfomycin MIC values by ≥ 4 -fold in 52% of the clinical strains tested. When PPF was combined with fosfomycin, a bacteriostatic or bactericidal effect was observed against representative *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* clinical strains but not against the highly resistant *E. coli* YD472 strain. These data suggest that PPF inhibited the function of FosA and, in doing so, restored the antibacterial activity of fosfomycin. In this regard, we hypothesize that the apparent lack of activity of PPF, at concentrations of up to 667 μM , against some clinical strains or transformants with high fosfomycin MIC values likely results from higher FosA protein levels, necessitating higher concentrations of PPF to inhibit these FosA proteins, and additional, non-*fosA*-mediated resistance mechanisms, such as defective transportation of fosfomycin across the inner membrane, in some instances. The role of sugar phosphate transporter defects in fosfomycin resistance is well documented for *E. coli in vitro*, but these mutations incur significant fitness costs, and their clinical relevance has not been established. In addition, expression of GlpT and UhpT is known to be elevated via activated fumarate nitrate reduction under anaerobic conditions, which confers increased antibacterial activity of fosfomycin (22). While our assays were conducted only under aerobic conditions, testing of the effect of PPF under anaerobic conditions might provide additional useful information. In *Enterobacteriaceae* species other than *E. coli*, mechanisms of fosfomycin resistance other than FosA production are poorly understood and require further studies.

There are several limitations to our study. First and foremost, significant reduction in fosfomycin MICs was not observed in 48% of the clinical strains tested. This result might be caused by insufficient concentrations of PPF, high-level fosfomycin resistance

at baseline, or the presence of additional resistance mechanisms such as transporter defects, as discussed above. This also suggests that potential clinical applications would need to be combined with testing of the effect of PPF on the fosfomycin MIC of the strain of interest. Second, the approach would not have benefit for infections caused by species that are intrinsically resistant to fosfomycin but lack *fosA*, such as *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* (9). Finally, PPF, although approved for clinical use, is known to have multiple toxicities, including nephrotoxicity, hypocalcemia, and seizures (23). Treatment duration for acute bacterial infections (7 to 14 days) will likely differ from that for the approved indications, such as CMV retinitis (21 to 28 days or longer), when used as an adjuvant for fosfomycin, which may alleviate some of the concerns regarding toxicity. However, this also suggests that additional toxicology studies are required to fully assess the feasibility of fosfomycin-PPF combinations, including proof-of-concept studies in an appropriate animal model.

In conclusion, we demonstrated the inhibitory activity of PPF against FosA in some MDR Gram-negative pathogens that produce chromosomal FosA, which is consistent with the robust competitive inhibition of FosA by this compound observed *in vitro*. Our findings suggest that inhibition of FosA activity may provide an opportunity to expand the usage of fosfomycin against Gram-negative species that produce FosA.

MATERIALS AND METHODS

Bacterial strains. Fosfomycin MICs were determined in 24 *K. pneumoniae*, 29 *E. cloacae*, and 16 *P. aeruginosa* strains, using the agar dilution method. Among these strains, 22 *K. pneumoniae*, 23 *E. cloacae*, and 15 *P. aeruginosa* strains that had fosfomycin MICs of >4 $\mu\text{g/ml}$ were included for evaluation of the inhibitory effect of PPF, using agar dilution testing. The following four clinical strains were used for the cloning of *fosA* genes, kinetic assays, and time-kill assays: *E. coli* YD472, *K. pneumoniae* I1, *E. cloacae* YDC666, and *P. aeruginosa* 75A7. *E. coli* YD472 is a previously reported strain that produces plasmid-encoded FosA3 along with CTX-M-65 extended-spectrum β -lactamase (ESBL) (24). *K. pneumoniae* I1 and *E. cloacae* YDC666 are carbapenem-resistant strains that produce KPC-type carbapenemase and were isolated from blood and a hematoma, respectively. *P. aeruginosa* 75A7 is a carbapenem-resistant strain from a urine culture. All strains were from clinical specimens obtained from patients at the University of Pittsburgh Medical Center.

Generation of *fosA* constructs and determination of FosA expression. The GenBank accession numbers for the *fosA* genes used in the study were [BAJ10054.1](#) for *E. coli* (*fosA3*), [YP_005224903.1](#) for *K. pneumoniae* (*fosA^{KP}*), [AIX57742.1](#) for *E. cloacae* (*fosA^{EC}*), and [NP_249820.1](#) for *P. aeruginosa* (*fosA^{PA}*). The four *fosA* genes were synthesized and cloned into pUC57 for susceptibility testing and into pE-SUMOstar (LifeSensors, Malvern, PA) for enzyme preparation (GenScript, Piscataway, NJ). The pUC57 constructs were introduced into *E. coli* TOP10 by electroporation. The pE-SUMOstar constructs were transformed into *E. coli* BL21(DE3) pLysS by heat shock. The FosA proteins were purified as described previously (8). Protein purity was assessed by SDS-PAGE, and protein concentrations were determined with the Bradford assay, using bovine serum albumin as the protein standard. Western blot analysis of FosA^{KP} and FosA^{PA} expression in *E. coli* TOP10 was performed by using anti-His₆ monoclonal antibody (Thermo). The details are described in the supplemental material.

Steady-state kinetic assays. Fosfomycin-dependent glutathione conjugation was detected spectrophotometrically using monochlorobimane (Sigma-Aldrich, St. Louis, MO), as described previously (8). The reaction was performed at 25°C in a total volume of 50 μl , in 0.1 M sodium phosphate buffer (pH 8.0) containing 50 mM KCl, 25 μM MnCl₂, 30 mM glutathione, and various concentrations of fosfomycin and PPF. Reactions were initiated by the addition of 100 nM FosA. Assays were run for 20 min (FosA3 and FosA^{KP}) or 30 min (FosA^{EC} and FosA^{PA}) before they were quenched by the addition of 150 μl methanol. The quenched reaction mixtures were then diluted 10-fold with 0.1 M sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 500 μM monochlorobimane. Glutathione concentrations were determined by fluorescence spectroscopy using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Data were analyzed by nonlinear regression using GraphPad Prism software (Systat Software, San Jose, CA). Data are reported as the mean $\pm$ standard error of at least three independent biological experiments.

Susceptibility testing. MICs of fosfomycin were determined with the agar dilution method according to CLSI guidelines, with Mueller-Hinton (MH) agar supplemented with 25 $\mu\text{g/ml}$ glucose-6-phosphate (16). *E. coli* ATCC 25922 was used as the quality control strain. The contribution of PPF to fosfomycin susceptibility was examined by the addition of 167 μM , 333 μM , or 667 μM PPF to MH agar plates. Inhibition by 1 mM and 5 mM PPF was also tested for *E. coli* YD472, *K. pneumoniae* I1, *E. cloacae* YDC666, and *P. aeruginosa* 75A7.

Time-kill assays. Time-kill assays were conducted using serial changes in the concentration of fosfomycin (0.5, 1, and 2 times the MIC) and 25 $\mu\text{g/ml}$ glucose-6-phosphate, with or without 667 μM PPF. The exception was *E. coli* YD472, for which we used fosfomycin concentrations of 256, 512, and 1,024 $\mu\text{g/ml}$ because of the high fosfomycin MIC of this strain ($>1,024$ $\mu\text{g/ml}$). Briefly, overnight isolated colonies of each strain were used to prepare a 0.5 McFarland standard inoculum in 0.85% NaCl. MH broth

was inoculated with 10^6 CFU/ml and incubated with shaking at 37°C. The numbers of viable CFU were determined at 0, 4, 8, and 24 h by serial dilution followed by culturing on MH agar plates. The numbers of colonies were counted after 24 h of incubation. A bacteriostatic effect and a bactericidal effect were defined as a decrease of $<3 \log_{10}$ CFU/ml and $\geq 3 \log_{10}$ CFU/ml, respectively, relative to the level of the starting inoculum.

Whole-genome sequencing. *E. coli* YD472, *K. pneumoniae* I1, *E. cloacae* YDC666, and *P. aeruginosa* 75A7 were subjected to whole-genome sequencing with a NextSeq system (Illumina, San Diego, CA). The reads were assembled *de novo* using the CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). We compared the amino acid alignment of FosA in those strains with those of FosA^{KP}, FosA^{EC}, FosA^{PA}, and FosA3, which were generated in this study. The genes associated with fosfomycin resistance (*murA*, *glpT*, *uhpT*, *uhpA*, *uhpB*, *uhpC*, *ptsI*, and *cyaA* genes) in *E. coli* YD472 were compared with those in the *E. coli* reference genome, *E. coli* K-12 (GenBank accession number U00096.3).

Assays of carbohydrate utilization by YD472. The overnight culture of *E. coli* YD472 in MH broth was collected by centrifugation. The pellet was washed twice and suspended in saline. The bacterial suspension was streaked onto M9 minimal medium agar containing 0.2% glucose-6-phosphate or glycerol-3-phosphate as the sole carbon source. The growth was observed after incubation at 35°C for 48 h. Strains lacking growth or showing poor growth without formation of colonies were considered deficient in transporter function.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01424-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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