Evaluation of Complexes of DNA Duplexes and Novel Benzoxazoles or Benzimidazoles by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry is used to compare the metal ion binding and metal-mediated DNA binding of benzoxazole (**1**, **2**, **3**, **4**) and benzimidazole (**5**) compounds and to elucidate the putative binding modes and stoichiometries. The observed metal versus non-metal-mediated DNA binding, as well as the specificity of DNA binding, is correlated with the biological activities of the analogs. The ESI-MS spectra for the antibacterial benzoxazole and benzimidazole analogs **4** and **5** demonstrated non-specific and non-metal-mediated binding to DNA, with the appearance of DNA complexes containing multiple ligands. The anticancer analog **2** demonstrates a clear preference for metal-mediated DNA interactions, with an apparent selectivity for Ni^{2+} -mediated binding over the more physiologically relevant Mg^{2+} or Zn^{2+} cations. Complexation between DNA and the biologically inactive analog **1** was not observed, either in the absence or presence of metal cations. (J Am Soc Mass Spectrom 2004, 15, 1593–1603) © 2004 American Society for Mass Spectrometry

There has been tremendous growth in recent years in the number and types of new DNA-interactive drugs developed, with an emphasis on creating drugs that have greater degrees of selectivity for specific DNA sequences or DNA structures [1–10]. If the drugs bind only to certain types of DNA or recognize cells that contain specific types of DNA, then the drugs can interfere with the transcription or replication of targeted DNA, thus exerting their pharmacological activities [1–10]. The drugs frequently bind to DNA in a noncovalent fashion, and thus methods to examine the nature of the interactions, the binding sites, and stoichiometry are needed. Electrospray ionization-mass spectrometry (ESI-MS) has been used to examine noncovalent drug/DNA complexes that are formed in solution and gently transported into the gas phase [11–40]. ESI-MS gives direct information about the stoichiometry of the noncovalent complexes, along with information about sequence selectivity and binding affinities, and it is proving to be a versatile way to screen new drugs for their DNA binding properties. In this report, we focus on the use of ESI-MS to evaluate several novel benzoxazole and benzimidazole analogs that bind to DNA.

The bis(benzoxazole) natural product UK-1, a secondary metabolite isolated from *Streptomyces*, has been shown to possess a potent anticancer activity toward a variety of cancer and tumor-derived cells [41, 42] (see Figure 1). In contrast, UK-1 was found to lack antibacterial activity against bacteria, yeast, and fungi. Previous studies have reported that UK-1 also binds a variety of divalent metal cations and also binds DNA in a metal-mediated manner in solution, in particular with the Mg^{2+} ion. The observation of metal-mediated DNA binding by UK-1 may be associated with its ability to serve as a catalytic inhibitor of human topoisomerase II [42, 43], which in turn may be related to the anticancer activity of this natural product [42]. We have previously evaluated the metal-mediated DNA binding of UK-1 using electrospray ionization mass spectrometry, in which complexes of the type $[\text{ds} + \text{UK-1} + \text{metal}]^{-n}$ were detected for solutions containing self-complementary oligodeoxynucleotides that formed duplexes (ds), and either Ni^{2+} , Co^{2+} , or Zn^{2+} cations [32].

Several simplified analogs of UK-1, various benzoxazole and benzimidazole compounds (Figure 1), have been prepared to investigate the basic structural requirements for metal ion binding, and to compare the antibacterial versus anticancer cytotoxicity activities of these analogs relative to that of UK-1 [42]. Of the analogs included in this study, **4** and **5** displayed a modest antibacterial activity against *Staphylococcus au-*

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Figure 1. Structures of benzoxazole and benzimidazole ligands.

reus, but lacked measurable cytotoxicities [42]. In contrast, one of the analogs, **2**, displayed a potent cytotoxicity against several cancer cell lines at a level comparable to UK-1 itself, although no significant antibacterial activity against *S. aureus* was observed [42].

In the present study, electrospray ionization mass spectrometry is used to detect noncovalent complexes containing oligodeoxynucleotides and the benzoxazole or benzimidazole analogs and to compare the metal ion binding and metal-mediated DNA binding with the known antibacterial and anticancer activities of the analogs. In each case, the observed metal versus non-metal-mediated DNA binding, as well as the specificity of DNA binding, is correlated with the biological activities of the analogs. Overall, the results for these experiments demonstrate that ESI-MS can be an efficient tool for screening DNA-interactive compounds, including the rapid determination of complex stoichiometries and binding specificities.

Experimental

Chemicals

The synthetic UK-1 analogs **2**, **4**, and **5** were prepared as previously reported [42]. **1** was purchased from Aldrich (Milwaukee, WI) and used without further purification. Methyl 2-(2'-hydroxyphenyl)-benzoxazole-7-carboxylate (**3**) was prepared by a modification of our previously reported benzoxazole synthesis [42]. The synthetic details for the latter are available as supplementary information.

Ammonium salts of HPLC-purified self-complementary oligodeoxynucleotides (ODNs) were purchased from TriLink Biotechnologies Inc. (San Diego, CA), custom synthesized on the 1.0 μ mol scale with purification by HPLC in an ammonium acetate buffer. Annealing was performed in 1 M ammonium acetate by heating to 90 °C for ten min, followed by slowly cooling to room temperature over 8 h. Solutions containing equimolar concentrations (25 μ M each) of one analog, one ODN, and one metal salt (where applicable) were

prepared in spray solvents containing 50% methanol in water with 50 mM ammonium acetate, and allowed to equilibrate at room temperature at nominally 6.5 pH prior to analysis. The metal salts were purchased from Aldrich Chemical.

Methods

Electrospray ionization mass spectra of the solutions were collected with a Finnigan LCQ Duo ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization source operated in the negative ionization mode with an electrospray voltage of 4 kV. The instrument response was optimized for maximum transmission of the bare ODN duplex ion using the autotune feature of the mass spectrometer. The analyte solution was introduced into the instrument by direct infusion at a flow rate of 3 μ L/min. A heated capillary temperature of 90 °C was used to ensure adequate desolvation while minimizing in-source dissociation of the noncovalent complexes. The base pressure of the trap was about 1.0×10^{-5} torr.

Cytotoxicity [44] and antimicrobial [42] assays were undertaken as described previously. Details are available in the supplementary section.

Results and Discussion

The DNA binding activities of the UK-1 analogs shown in Figure 1 were evaluated by ESI-MS to determine and compare the stoichiometries and metal ion requirements of gas-phase complexes with what is known for these compounds in solution. The results were also compared with the observed antibacterial versus anticancer activities of the analogs.

Complexation of DNA and UK-1 Analogs Without Metals

A series of experiments were performed in which ESI-MS was used to evaluate the complexation between the UK-1 analogs and self-complementary oligodeoxynucleotides (ODNs) in the absence of metals. The use of self-complementary ODNs produces readily interpretable mass spectra with peaks generally composed of single ion species [16, 17]. The unambiguous assignment of even charge state duplex ions cannot be made due to overlap of the m/z ratios for duplex ions and single-stranded ions of half the charge. However, the m/z value and charge state can be indirectly determined from examination of the metal adduct peaks, provided that sufficient resolution of these metal adducts from the parent is achieved. The charge states and hence m/z values of complexes containing an odd multiple of drug, metal, or drug plus metal can be assigned unambiguously for even charge state complexes due to non-overlapping m/z with the corresponding single-strand complexes.

Figure 2. ESI-mass spectra for solutions containing 5'-GCGAATTCGC-3' and (a) 1, (b) 2, (c) 4, and (d) 5 analogs.

For example, the electrospray mass spectrum of the self-complementary oligodeoxynucleotide 5'-GCGAATTCGC-3' sprayed from a solution containing 25 μ M DNA in 25% methanol and 50 mM ammonium acetate exhibits an ion at m/z 1210 which can be unambiguously assigned as $[\text{ds} - 5\text{H}]^{-5}$ due to its odd charge state (see Figure 2a). However, the even charge state for the ion at m/z 1513 indicates this ion could represent either the $[\text{ss} - 2\text{H}]^{-2}$ or $[\text{ds} - 4\text{H}]^{-4}$ species, or possibly a mixture of the two. Likewise, the ion at m/z 1008 could be $[\text{ss} - 3\text{H}]^{-3}$ or $[\text{ds} - 6\text{H}]^{-6}$, although expansion of this ion and examination of the sodium adduct satellite peaks suggests that the identity is $[\text{ss} - 3\text{H}]^{-3}$. Although apparent duplex ions could result from non-specific aggregation of single strands during the electrospray process, this has previously been shown to be minimized or eliminated under similar solvent conditions and ODN concentrations as was used in this study [16, 17]. Thus, for the studies presented here, the duplex DNA peaks observed in the electrospray mass spectra are assumed to result from specific association of the self-complementary strands.

Two of the analogs in Figure 1, 4 and 5, have previously been shown to exhibit modest antibacterial activities against two strains of *S. aureus*, but lack any significant cytotoxicities against four cancer cell lines [42]. Although the specific antibacterial mechanisms of these analogs are unknown, inhibition of bacterial gyrase consequent upon binding to either DNA alone or to the DNA-gyrase complex is suspected, probably in a

manner similar to what has been proposed for the quinolone class of antibiotics [45–48].

Solutions containing one self-complementary duplex ODN and one analog were prepared in 25% methanol containing 50 mM ammonium acetate, and mass spectra were collected in the negative ion mode. As shown in Figure 2 for solutions containing 5'-GCGAATTCGC-3' and each analogue, a distribution of complexes of both -4 and -5 charge states and containing from one to four drug molecules per DNA duplex were detected in modest abundance for each of the antibiotic 4 and 5 analogs (Figure 2c and d, respectively). Based on experiments in which the concentration of the drug ligand was increased or decreased by a factor of five relative to the fixed concentration of DNA (data not shown), the total number of bound drugs was found to be dependent upon the overall drug:DNA ratio, indicative of intercalative or non-specific binding without any well-defined stoichiometry.

The lack of a well-defined DNA binding stoichiometry by the antibiotic analogs is reminiscent of results reported by Gabelica et al. for drug-DNA complexes containing known intercalators such as ethidium bromide, amsacrine, and ascididemin [16]; as well as Kapur et al. for the more complex intercalators daunomycin and nogalamycin [36]. In the study by Gabelica, the DNA-binding modes observed in the gas-phase for ethidium bromide, amsacrine, and ascididemin exhibited the non-specific multiple-drug binding which parallels the binding behaviors observed in solution [16].

Figure 3. ESI-mass spectrum of solution containing norfloxacin and 5'-GCGAATTCGC-3'.

However, the relative peak intensities also paralleled ligand polarity, including ligand charge, and thus the observed binding pattern could simply be an artifact of the electrospray process. The peak distribution patterns reported by Kapur et al. for daunomycin and nogalamycin were interpreted as following the neighbor exclusion principle predicted for intercalators, in which one drug molecule is inserted between every other base pair in the duplex DNA [36].

In contrast to the results described above for the antibiotic analogs, spectra collected for solutions containing the same ODN and the inactive analog **1** or **3** or the anticancer analog **2** showed no detectable drug-DNA complexes under identical ESI-MS and spray solvent conditions. Representative ESI-mass spectral results are shown in Figure 2a and c for the duplex ODN with **1** and **2**, respectively (similar data for **3** is not shown). Thus, the **1**, **2**, and **3** compounds are useful as negative controls for monitoring electrospray-induced artifacts such as aggregation or random electrostatic binding.

Although the specific DNA binding mode or modes for the antibacterial analogs cannot be unambiguously determined from these results, it seems reasonable that the benzyl substituents must contribute to the observed binding behavior, possibly by additional stabilization of the complexes through ring stacking or partial insertion of the benzyl groups between DNA base pairs. Although these results suggest an intercalative binding mode, other modes including minor or major groove binding, or a possible mixture of groove and intercalative modes, cannot be ruled out. Results from recent spectroscopic and molecular modeling studies by Lee et al. have indicated a mixed binding mode incorporating both groove binding and partial intercalation for interactions between the quinolone antibiotic norfloxacin and DNA [49, 50].

For comparison, solutions containing the same self-complementary 10-mer 5'-GCGAATTCGC-3' and norfloxacin were prepared, and mass spectra collected under the same conditions as for the analogs described

above. The spectrum in Figure 3 shows a nearly identical peak distribution as observed for the **4** and **5** antibiotic analogs. In contrast, spectra for solutions containing the quinolone antibiotic nalidixic acid, which exhibits a much lower antibacterial activity than norfloxacin, exhibited no detectable formation of drug-DNA complexes.

DNA and UK-1 Analogs with Metals

Previous studies by Reynolds et al. and Kumar et al. have implicated the role of divalent metal cation binding in the anticancer activities of UK-1 [41, 42]. In these studies, both UK-1 and the anticancer analog **2** were observed to form monomeric 1:1 complexes with divalent metal cations in solution, in particular with the physiologically relevant cations Mg^{2+} and Zn^{2+} . In addition, fluorescence and spectrophotometric experiments have shown that the double-stranded DNA-binding activity of UK-1 is ten-fold higher in the presence of Mg^{2+} , thus indicating that DNA binding by UK-1 is metal mediated [41]. The metal-mediated binding of UK-1 to DNA has also been observed in our laboratory by ESI-MS, although the results demonstrate a clear preference for Ni^{2+} , Co^{2+} , and Zn^{2+} -mediated binding, as opposed to Mg^{2+} in which no detectable (DNA + UK-1 + Mg^{2+}) complexes were observed in the electrospray mass spectra [32].

Given the implication for metal binding in the mechanism of action of UK-1 and the analogs, the metal-mediated DNA-binding activities of the analogs was also evaluated by electrospray ionization (Figure 4). Solutions containing the antibacterial analogs (**4** and **5**) and divalent metal cations, such as Ni^{2+} , produced no obvious enhancement of DNA binding, nor significant changes in peak distributions other than the presence of low abundance species corresponding to metal-adducted ions (as expected). Therefore, this indicates a

Figure 4. ESI-mass spectra of solutions containing 5'-GCGAATTCGC-3' and Ni^{2+} with (a) 4, (b) 2, and (c) 1.

non-metal-mediated DNA binding mode for the anti-bacterial analogs.

In contrast, the anticancer analog 2 showed a marked enhancement in the formation of DNA-drug-metal

complexes in the presence of Ni^{2+} (Figure 4b), and a modest enhancement in the presence of Cu^{2+} . Interestingly, no significant binding was observed upon ESI-MS analysis of solutions containing 2 and the more

Figure 5. ESI-mass spectra of solutions containing 5'-GCGAATTCGC-3' and 2 with (a) Mg^{2+} and (b) Ni^{2+} .

Figure 6. ESI-mass spectra of solutions containing 5'-GCGAATTCGC-3' and **3** with (a) Mg²⁺ and (b) Ni²⁺.

physiologically relevant Mg²⁺ and Zn²⁺ cations, nor with any of the other metals listed above (Figure 5). The same experiments performed with the biologically inactive analog **1** revealed a complete lack of DNA binding, both in the presence and absence of divalent metal cations, providing once again a negative control for non-specific binding (Figure 4c). Since the **1** and **2** analogs differ only by the 4-carbomethoxy group in **2**, the DNA binding results indicate that this group is obligatory for the observed metal-mediated binding behavior.

The metal-mediated DNA complexation of **3** was also examined by ESI-MS. DNA-**3**-metal complexes of very low intensities were observed in the presence of Mg²⁺ or Ni²⁺ (see Figure 6), indicating both less selectivity and much lower affinity than that observed for **2**. The relative intensity of the DNA-**3**-Ni²⁺ complexes is about ten to forty times less than that observed for the analogous **2** ligand.

NMR Studies

We have also carried out solution NMR studies of the metal ion binding of **2** and its reversed analog **3**. Solutions of **2** in CD₃OD/CDCl₃ (100 mM) were prepared and all ¹H resonances were assigned by a combination of COSY, HMBC, and HMQC NMR spectra. The **2** solution was titrated with Mg(NO₃)₂·(H₂O)₆ in CD₃OD (2.5 M) and the ¹H NMR spectra recorded after

each addition (Figure 7a). The initial addition of Mg²⁺ (0.25 eq) caused a marked broadening of all the ¹H NMR resonances and shifts in specific peaks. Notably, certain resonances, such as that due to the benzoxazole C₇ hydrogen (ca. 7.5 ppm), shifted downfield while other resonances, such as those due to the 4-carbomethoxy group (ca. 3.7 ppm) shifted upfield. Further additions of Mg²⁺ resulted in upfield shifts of all resonances, but in particular those due to the 4-carbomethoxy group and the *ortho*-hydroxyphenyl moiety (ca. 6.7 and 6.8 ppm).

These solution studies support the role of the 4-carbomethoxy group in metal binding by **2** and also implicate the benzoxazole moiety and the *ortho*-hydroxyphenyl substituent in metal ion coordination. The complex relationship between ¹H NMR resonance shifts and Mg²⁺ concentration may be due to the formation of different metal ion complexes of different stoichiometries during the titration experiment. Unfortunately, attempts to shed light on potential differences in structure between the complexes formed with **2** and Mg²⁺ or Ni²⁺ by NMR titrations are precluded by the paramagnetic properties of the latter. NMR titrations of **2** with various Cu²⁺ salts also resulted in severe line broadening, presumably due to formation of paramagnetic Cu⁺ species.

The role of the benzoxazole moiety in metal ion coordination by **2** was explored by examining the Mg²⁺ binding of **3**, the reverse isomer of **2** in which the

Figure 7. ^1H NMR spectrum of (a) **2** in the presence of Mg^{2+} and (b) **3** in the presence of Mg^{2+} .

position of the benzoxazole N and O atoms are interchanged. The ^1H NMR spectral changes observed upon addition of Mg^{2+} to the isomeric benzoxazole analog **3** (Figure 7b) are distinct from those observed for **2**. The ^1H NMR resonances for **3** are uniformly shifted upfield

in the presence of increasing concentrations of Mg^{2+} , with no evidence for line broadening until an excess of Mg^{2+} is added. The apparent difference in the metal ion complexation between the **2** and **3** must be due to the difference in the disposition of the benzoxazole N and

Figure 8. ESI-mass spectrum of a solution containing **2** and Ni^{2+} .

O atoms, further supporting a role for the benzoxazole moiety in Mg^{2+} coordination by **2**.

Metal Binding of **2** and **3**

Given the marked dependence upon the presence of divalent metal cations for binding between DNA and analog **2**, we also investigated the drug-metal interactions by ESI-MS in order to gain insight into the differences in drug-metal binding modes, which may in turn provide clues to the observed gas-phase metal-mediated DNA binding behavior described above. These experiments were also done to provide insight into the binding between **2** and metal cations not accessible by NMR spectroscopy. The metal-mediated binding properties of analog **2**, and its reverse analog **3**, were evaluated in the absence of DNA using similar spray solvent and instrument conditions as used for the drug-DNA binding studies above, but with spectra collected in the positive ion mode.

Solutions containing **2** or **3** and one of several different metal salts were prepared and analyzed by ESI-MS. Two dominant ions were observed for solutions containing **2** (or **3**) with Mg^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} or Ni^{2+} : protonated **2** and the singly charged dimer species assigned as $[\mathbf{2} \times \mathbf{2} - \text{H} + \text{M}^{2+}]^+$, in which M^{2+} is the metal ion (see Figure 8). There is no apparent metal selectivity in the formation of the dimeric complexes; however, collisional activated dissociation of the complexes revealed a striking difference in the fragmentation pathways of the metal complexes (see Figure

9). For the complexes containing **2** and Mg^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , or Zn^{2+} , the two major fragmentation pathways involve loss of one neutral **2** ligand with rapid adduction of water, or loss of methanol (Figure 9a). For the complexes containing **2** and Cu^{2+} or Ni^{2+} , the only dissociation pathway is the loss of a neutral **2** ligand with rapid adduction of water (from reaction with traces of background water in the trap), and the elimination of methanol is not observed (Figure 9b).

The fact that the methanol elimination pathway is completely quenched for the Ni^{2+} and Cu^{2+} complexes suggests a significant change in the structures of these dimeric structures. The loss of methanol from the $[\mathbf{2} \times \mathbf{2} - \text{H} + \text{M}^{2+}]$ dimers likely involves a methoxy group from one ligand and a hydrogen from the second ligand, thus requiring an intramolecular migration in which the two moieties (methoxy and hydrogen) must be in sufficiently close proximity to facilitate the elimination. In the Ni^{2+} complexes, the two **2** ligands may be rotated in such a way that the methanol loss can not occur. Although these dimeric species are not necessarily involved in the formation of the DNA complexes, it does suggest that Ni^{2+} coordinates to **2** in an unusual way, and this unique Ni^{2+} selectivity may be responsible for mediating the complexation to DNA.

Similar CAD studies were carried out for the dimer containing the reverse analog, **3**. For both the $[\mathbf{2} \times \mathbf{3} - \text{H} + \text{Mg}^{2+}]^+$ and $[\mathbf{2} \times \mathbf{3} - \text{H} + \text{Ni}^{2+}]^+$ complexes, the loss of methanol is never observed upon activation of the complexes. Instead the dimeric complexes dissociate by elimination of one **3** ligand, with rapid adduction of water to the resulting product ions. The complete

Figure 9. CAD mass spectrum of (a) $(2 \times 2 - H + Mg^{2+})^+$ and (b) $(2 \times 2 - H + Ni^{2+})^+$.

absence of the loss of methanol suggests that the phenolic hydroxyl group and methyl group are never in sufficiently close proximity to facilitate the loss of methanol from the dimeric complexes.

Biological Studies

As previously reported, UK-1 and the benzoxazole analog **2** are cytotoxic to cancer cell lines, particularly PC-3 prostate cancer cells ($IC_{50} = 0.4$ and $0.91 \mu M$, respectively) [42]. Neither of these compounds are active as antibacterial agents [42], whereas the benzimidazole **5** and benzoxazole **4** are weakly antibacterial (IC_{50} *S. aureus* = 102 and $43 \mu M$, respectively). The cancer cell cytotoxicity and antibacterial activity of the previously unreported UK-1 analog **3** were determined. Like UK-1 and **2**, **3** is inactive against *Staphylococcus aureus* and methicillin-resistant *S. aureus* at all test concentrations, up to $185 \mu M$. **3** displayed only weak cytotoxicity against PC-3 prostate cancer cells ($IC_{50} = 47 \mu M$). This level of cytotoxicity is more than 50-fold less than that of the isomeric benzoxazole **2**, and over 100-fold weaker than the natural product UK-1. **3** was also only weakly active against the colon cancer cell line HT-29 ($IC_{50} = 36 \mu M$), as compared to the isomeric benzoxazole **2** ($IC_{50} = 9.1 \mu M$). Thus, the cancer cell cytotoxicity of these benzoxazole analogs of UK-1 is strongly sensitive to their structural features, and mirrors their relative ability to form DNA-drug-metal complexes.

Conclusions

The results presented in this study demonstrate the use of ESI-MS to evaluate the interactions between the UK-1 analogs and DNA, in particular to gain information on the putative binding modes, stoichiometries, and metal ion requirements. The ESI-mass spectra for the antibacterial benzoxazole and benzimidazole analogs **4** and **5** demonstrated non-specific and non-metal-mediated binding to DNA, with the appearance of multiple-drug peaks analogous to binding observed for the quinolone antibiotic norfloxacin, and perhaps reminiscent of binding patterns observed for simple intercalators.

In contrast, ESI-MS of the DNA binding by the anticancer analog **2** demonstrates a clear preference for metal-mediated DNA interactions, with an apparent selectivity for Ni^{2+} -mediated binding over the more physiologically relevant Mg^{2+} or Zn^{2+} cations. No gas-phase noncovalent complexes between DNA and the biologically inactive analog **1** were detected, either in the absence or presence of metal cations. Consequently the **1** analog provides a useful negative control compound for relating observed DNA binding by ESI-MS and biological activity, as well as controlling for random electrostatic DNA binding and electrospray artifacts such as non-specific aggregation. Overall, the observed binding and metal ion requirements for interactions between DNA and the simpler UK-1 analogs correlate with their known antibacterial versus anticancer activities.

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Appendix Supplemental Material

Supplemental material associated with this article can be found, in the online version, at doi:10.1016/j.jasms.2004.07.015.

Supplemental material available: Description of the synthetic preparation of **3** and assays.

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