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Activity of Quinolone CP-115,955 Against Bacterial and Human Type II Topoisomerases Is Mediated by Different Interactions

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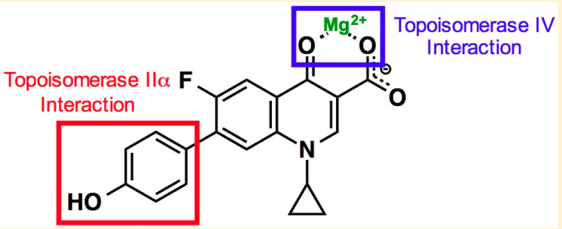
Supporting Information

ABSTRACT: CP-115,955 is a quinolone with a 4-hydroxyphenyl at C7 that displays high activity against both bacterial and human type II topoisomerases. To determine the basis for quinolone cross-reactivity between bacterial and human enzymes, the activity of CP-115,955 and a series of related quinolones and quinazolinediones against *Bacillus anthracis* topoisomerase IV and human topoisomerase IIα was analyzed. Results indicate that the activity of CP-115,955 against the bacterial and human enzymes is mediated by different interactions. On the basis of the decreased activity of quinazolinediones against wild-type and resistant mutant topoisomerase IV and the low activity of quinolones against resistant mutant enzymes, it appears that the primary interaction of CP-115,955 with the bacterial system is mediated through the C3/C4 keto acid and the water−metal ion bridge. In contrast, the drug interacts with the human enzyme primarily through the C7 4-hydroxyphenyl ring and has no requirement for a substituent at C8 in order to attain high activity. Despite the fact that the human type II enzyme is unable to utilize the water−metal ion bridge, quinolones in the CP-115,955 series display higher activity against topoisomerase IIα *in vitro* and in cultured human cells than the corresponding quinazolinediones. Thus, quinolones may be a viable platform for the development of novel drugs with anticancer potential.



All organisms encode type II topoisomerases. These enzymes alter DNA topology by generating a transient double-stranded break in the genetic material and passing a separate double helix through the DNA gate.[1−6] Humans as well as most bacterial species encode two type II topoisomerases—topoisomerase IIα and IIβ in humans, and gyrase and topoisomerase IV in bacteria.[1−6] In addition to their critical physiological functions, these enzymes are important drug targets.

Human type II topoisomerases are targeted by a number of widely prescribed anticancer agents, including etoposide, doxorubicin, mitoxantrone, and amsacrine.[5,7,8] These drugs act as topoisomerase II poisons and increase levels of enzyme-mediated DNA cleavage. Ultimately, they kill cells by increasing the concentration of topoisomerase II-linked DNA strand breaks to the point that they overwhelm repair processes and trigger apoptosis.[5,7,8] Quinolones, including ciprofloxacin and moxifloxacin, are broad-spectrum antibacterial agents that are used to treat a wide variety of Gram-negative and Gram-positive infections.[5,9−13] Similar to the anticancer drugs, quinolones act as poisons and kill bacteria by increasing levels of DNA strand breaks generated by gyrase and topoisomerase IV.[10,13−18]

Clinically relevant quinolones interact with bacterial type II enzymes primarily through a water−metal ion bridge, which was first characterized in *Bacillus anthracis* topoisomerase IV.[19,20] This water−metal ion bridge is formed when the C3/C4 keto acid of the drug chelates a divalent metal ion, which is stabilized by four water molecules.[21] Two of these water molecules are coordinated by Ser81 and Glu85 (residues numbered by *B. anthracis* positions) in the GrlA subunit of topoisomerase IV. Mutations in these amino acid residues that anchor the water−metal ion bridge are the most prevalent cause of quinolone resistance.[10,13,15,16,18,22−31]

Human type II enzymes lack the serine and acidic amino acids necessary to anchor the water−metal ion bridge and are unable to support bridge function.[32] This is why clinically relevant quinolones display virtually no activity against human type II topoisomerases in the therapeutic range.[32]

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In the early 1990s, a new group of experimental quinolones, the CP-115,953/CP-115,955 series, was described.[33−37] Unlike any clinically relevant quinolone, these drugs contain a 4-hydroxyphenyl group at the C7 position (otherwise, CP-115,955 is structurally identical to ciprofloxacin) (Figure 1). In marked contrast to previous quinolones, CP-115,953 and CP-115,955 displayed high activity against both eukaryotic and bacterial type II topoisomerases.[19,32−39] This property allowed the first direct comparison of drug mechanism across phylogenic domains.[19,32−39]

Recently, another group of quinolones with high activity against bacterial[19,20,32,40] and human[32] type II topoisomerases has been described. These quinolones contain a 3′-(aminomethyl)pyrrolidinyl [3′-(AM)P] group at C7. As above, this C7 group is not represented in any clinically relevant quinolone. The 3′-(AM)P group (and related C7 groups) was reported previously as part of a series of quinazolinediones that displayed high activity against wild-type and quinolone-resistant bacteria.[41,42] Quinazolinediones (Figure 1) share structural homology with quinolones, but lack the C3/C4 keto acid that is required for metal ion chelation. As a result, they are unable to interact with bacterial type II topoisomerases through the water−metal ion bridge. A series of functional studies strongly suggest that 3′-(AM)P quinazolinediones increase DNA cleavage mediated by *B. anthracis* topoisomerase IV because this C7 group forms novel interactions within the enzyme-cleaved DNA complex.[32] These interactions are sufficient to allow the drugs to function against bacterial type II enzymes that contain mutations in the bridge-anchoring residues.[19,20,32,40,43]

A recent study[32] demonstrated that the 3′-(AM)P group was able to mediate drug interactions within covalent enzyme-cleaved DNA complexes formed with topoisomerase IIα. As a result, several quinolones and quinazolinediones that contain a C7 3′-(AM)P moiety display high activity against the human enzyme.[32]

To further understand the basis for quinolone cross-reactivity between bacterial and human systems, the effects of CP-115,955 on DNA cleavage mediated by *B. anthracis* topoisomerase IV and human topoisomerase IIα were analyzed. Results indicate that the activity of CP-115,955 against the bacterial and human type II topoisomerases is mediated by different interactions. Whereas the drug interacts with the bacterial enzyme primarily through the C3/C4 keto acid and the water−metal ion bridge, it interacts with the human enzyme primarily through the C7 4-hydroxyphenyl ring. Furthermore, the hydroxyphenyl moiety forms tighter interactions within topoisomerase IIα-cleaved DNA complex than does the 3′-(AM)P group. Finally, despite the fact that the human type II enzyme is unable to utilize the water−metal ion bridge, quinolones display higher activity against topoisomerase IIα *in vitro* and in cultured human cells than the corresponding quinazolinediones. Thus, quinolones may be a viable platform for the development of novel drugs with anticancer potential.

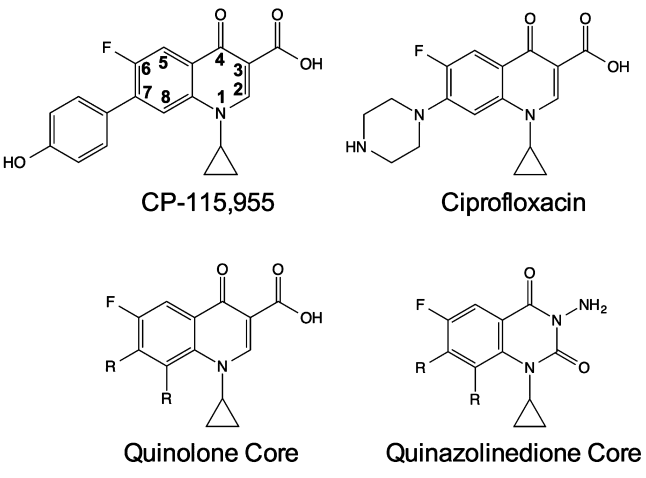

Figure 1. Structures of CP-115,955, ciprofloxacin, and the quinolone and quinazolinedione cores. CP-115,953 is identical to CP-115,955, except that it includes a fluorine at the C8 position.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Wild-type *B. anthracis* GrlA and GrlB and drug-resistant GrlA^{S81F} and GrlA^{S81Y} were expressed and purified as described previously.[20,44] The proteins were stored at −80 °C in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 20% glycerol. In all assays, topoisomerase IV was used as a 1:1 mixture of GrlA:GrlB.

Wild-type and mutant human topoisomerase IIα were expressed in *Saccharomyces cerevisiae* as described previously.[45] The wild-type enzyme was purified as described by Kingma et al.[46] Mutant human topoisomerase IIα containing M762S/M766E (hTop2A$^{M762S/M766E}$) was purified as described by Aldred et al.[32] Wild-type topoisomerase IIα also was prepared by this method and displayed activities that were nearly identical to enzymes prepared as described above by the protocol of Kingma et al.[32,45]

Negatively supercoiled pBR322 plasmid DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer.

Ciprofloxacin was obtained from LKT Laboratories, and CP-115,955 was the gift of Thomas D. Gootz and Paul R. McGuirk (Pfizer Global Research). Both compounds were stored at −20 °C as 40 mM stock solutions in 0.1 N NaOH and diluted 5-fold with 10 mM Tris−HCl (pH 7.9) immediately prior to use. Moxifloxacin was obtained from LKT Laboratories and was stored at 4 °C as a 20 mM stock solution in 100% DMSO. Etoposide was obtained from Sigma and stored at RT as a 20 mM stock solution in 100% DMSO. All other quinolones and quinazolinediones were synthesized using established methods as reported previously by German et al.[47,48] and Malik et al.,[47,48] and by Hashimoto et al.[49] and Zhang et al.[50] for incorporating the hydroxyphenyl ring at the C7 position. The syntheses and characterizations of these compounds are provided in the Supporting Information. 8-Methyl- and 8-methoxy-955 were stored at −20 °C as 40 mM stock solutions in 0.1 N NaOH and diluted 5-fold with 10 mM Tris−HCl (pH 7.9) immediately prior to use. Quinazolinediones were stored at 4 °C as 20 mM stock solutions in 100% DMSO. Table S1 in Supporting Information contains the full chemical, library, and abbreviated names of the compounds used in this study. All chemicals were analytical reagent grade.

DNA Cleavage Mediated by Topoisomerase IV. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff[19] as modified by Aldred et al.[19,51] Reactions contained 75 nM wild-type or mutant topoisomerase IV and 10 nM negatively supercoiled pBR322 in a total of 20 μL of 40 mM Tris−HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, and 2.5% (v/v) glycerol. Reaction mixtures were incubated at 37 °C for 10 min, and enzyme−DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS

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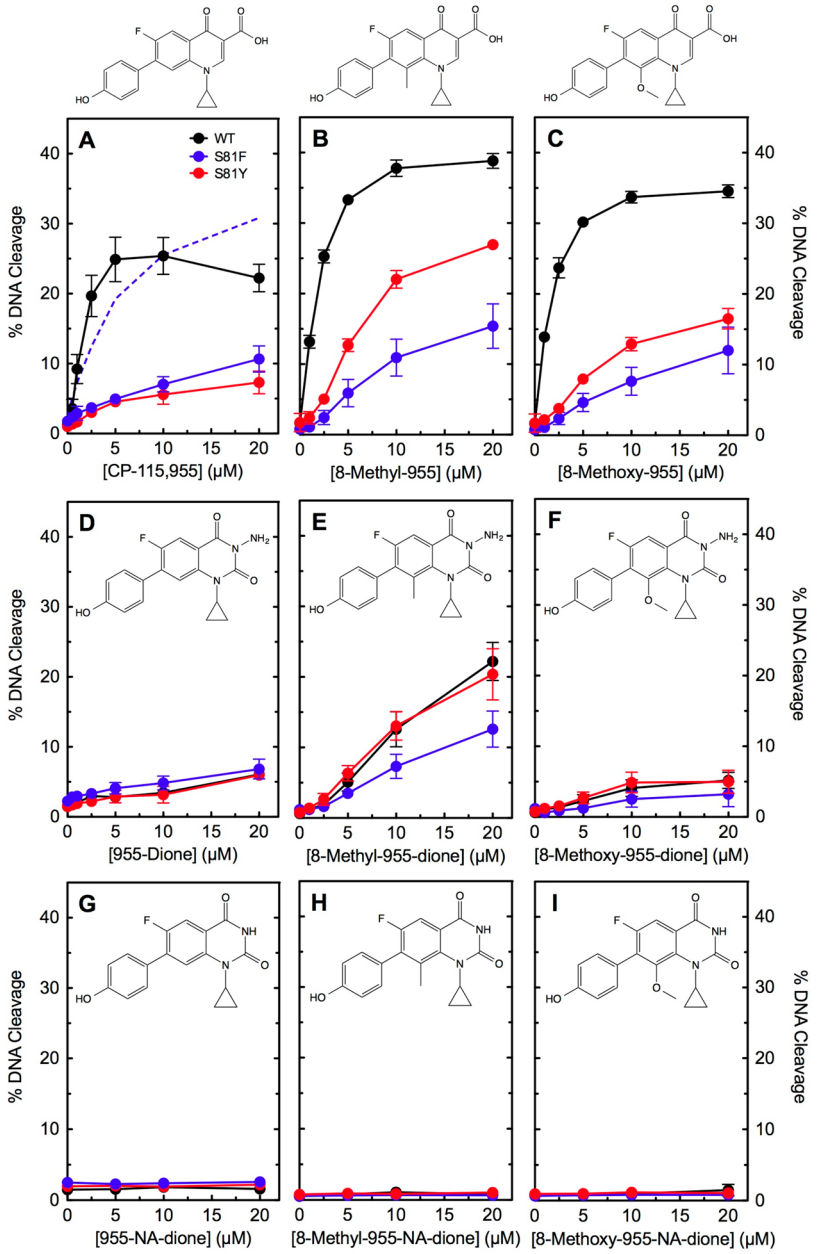

Figure 2. Effects of a CP-115,955-based series of quinolones, quinazolinediones, and non-amino-quinazolinediones on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type (WT, black), GrlA^{S81F} (S81F, blue), and GrlA^{S81Y} (S81Y, red) topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top row, panels A–C), quinazolinediones (middle row, panels D–F), or non-amino-quinazolinediones (bottom row, panels G–I) containing a C7 4-hydroxyphenyl group and a C8 hydrogen (left column, panels A, D, and G), methyl (middle column, panels B, E, and H), or methoxy (right column, panels C, F, and I) group is shown. Drug structures are shown above or in the respective panels. For comparison, the activity of 8-H-3′-(AM)P-FQ against GrlA^{S81F} (dashed blue line) is shown in the top left panel. Error bars represent the standard deviation of at least three independent experiments.

followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 45 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer [60% sucrose, 10 mM Tris–HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris–acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized with medium-range ultraviolet light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

Modeling Studies. Modeling studies were carried out as described by Drlica et al.[52] An initial manual analysis and manipulation of fluoroquinolone-topoisomerase IV cleavage complexes was performed using structures obtained from PBD accession number 2XKK for *Acinetobacter baumannii* topoisomerase IV[21] using WebLab ViewerLight. For computer-aided docking, SYBYL-X 1.3 was used to prepare the 2XKK crystal structure for ligand docking.[53] Standard Tripos procedures were followed for SYBYL-X 1.3 (Certara, L.P.).

DNA Cleavage Mediated by Human Topoisomerase IIα. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff[32] as modified by Aldred

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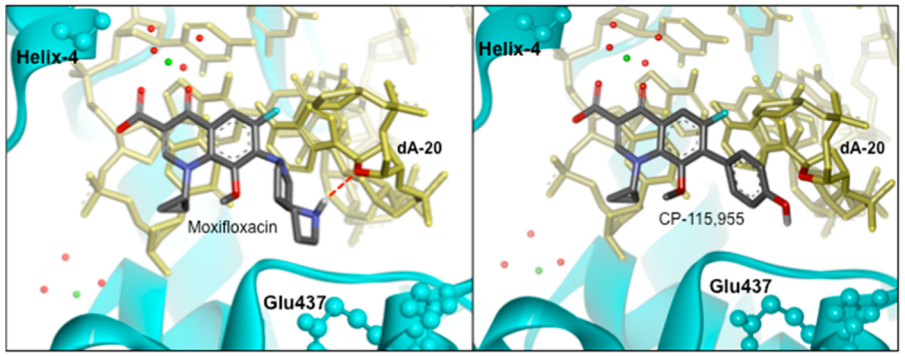

Figure 3. Docking of a CP-115,955 analog into the cleavage complex. The left panel shows the crystal structure of a moxifloxacin–*A. baumannii* topoisomerase IV cleavage complex.[21] The right panel shows the predicted structure of a cleavage complex in which moxifloxacin has been replaced by 8-methoxy-955. Quinolones are colored by atom type. The noncatalytic Mg^{2+} ion that is chelated by the C3/C4 keto acid of the quinolones and participates in the water–metal ion bridge interaction is shown in green. The four water molecules that fill out the coordination sphere of the Mg^{2+} ion are shown in red. The amino acid backbone of the enzyme is shown as ribbons. The hydrogen bond formed between the C7 diazabicyclononyl ring of moxifloxacin and dA20 is shown in red. Glu437, which is conserved in topoisomerase IV, is indicated. Quinolones and quinazolinediones that contain a C7 3′-(AM)P group overcome resistance by forming an interaction with this amino acid residue. No interactions are observed between the protein and the C7 4-hydroxyphenyl group of 8-methoxy-955.

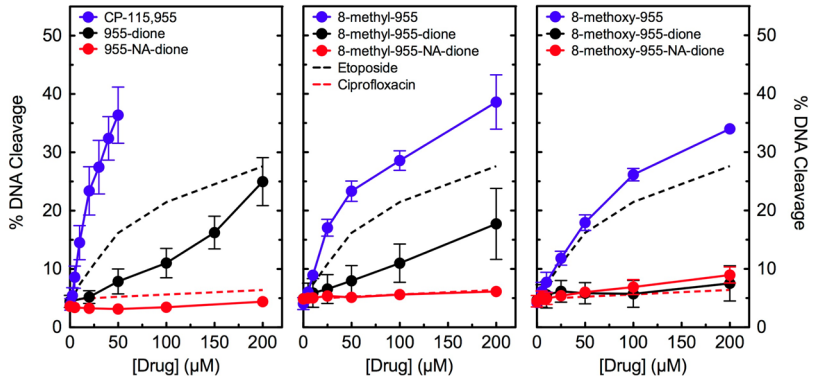

Figure 4. Effects of a CP-115,955-based series of quinolones, quinazolinediones, and non-amino-quinazolinediones on the DNA cleavage activity of human topoisomerase IIα. The ability of the enzyme to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (blue), quinazolinediones (black), or non-amino-quinazolinediones (red) containing a C7 4-hydroxyphenyl group and a C8 hydrogen (left), methyl (middle), or methoxy (right) group is shown. Results with etoposide (dashed black line) and ciprofloxacin (dashed red line) are shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

et al.[32,51] Reactions contained 110 nM wild-type or mutant (hTop2A$^{M762S/M766E}$) topoisomerase IIα and 10 nM negatively supercoiled pBR322 in a total of 20 μL of human cleavage buffer [10 mM Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 100 μM EDTA, 25 μM dithiothreitol, and 2.5% (v/v) glycerol]. Reaction mixtures were incubated at 37 °C for 10 min and processed as described above for topoisomerase IV plasmid DNA cleavage.

For assays that monitored competition between two drugs, the compounds were added simultaneously to reaction mixtures, and the final concentrations of the compounds are indicated. In these competition assays, the level of cleavage seen with the corresponding concentration of the competing drug (in the absence of the drug held at a constant concentration) was used as a baseline and was subtracted from the cleavage level seen in the presence of both compounds.

Persistence of Topoisomerase IIα-DNA Cleavage Complexes. The persistence of topoisomerase IIα–DNA cleavage complexes established in the presence of drugs was determined using the procedure of Gentry et al.[19] as modified by Aldred et al.[19,54] Initial reactions contained 550 nM wild-type or mutant hTop2A$^{M762S/M766E}$ enzyme, 50 nM DNA, and 20 μM CP-115,955 or 200 μM 955-dione in a total of 20 μL of human cleavage buffer. Reactions were incubated at 37 °C for 10 min and then diluted 25-fold with human cleavage buffer warmed to 37 °C. Samples (20 μL) were removed at times ranging from 0 to 1 min, and DNA cleavage was stopped with 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Samples were digested with proteinase K and processed as described above for cleavage assays. Levels of DNA cleavage were set to 100% at time zero, and the persistence of cleavage complexes was determined by the decay of the linear reaction product over time.

Intracellular Cleavage Complex Formation. Levels of topoisomerase IIα cleavage complexes formed in human CEM cells upon drug treatment were measured using the rapid approach to DNA adduct recovery (RADAR) assay[55] with modifications. Cells were grown in RPMI 1640 + L-glutamine culture medium supplemented with 10% bovine growth serum. A total of 600 000 cells were seeded at a density of 150 000 cells/mL in six-well cell culture plates. Following a 24 h incubation at 37 °C, cells were treated with drugs for 1 h at 37 °C. Cells were harvested by centrifugation (500*g* for 5 min at room temperature) and lysed with 4 M guanidine thiocyanate, 20 mM Tris-HCl, 20 mM EDTA, 2% Triton X-100, 1% Sarkosyl, 1% DTT, and 100 mM NaOAc, at pH 6.5 (personal

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communication with Kostantin Kiianitsa, University of Washington). Lysates were sheared with four passages through a 21-gauge needle and ethanol precipitated as described[55] for 20 min. Precipitated DNA was pelleted by centrifugation at 18000*g* for 20 min at 4 °C. Pellets were washed and resuspended in 8 mM NaOH as described.[55] DNA solutions were subjected to at least eight shearing passes through a 21-gauge needle. Levels of DNA-topoisomerase IIα cleavage complexes were determined by immunoblotting 0.5 μg of genomic DNA for topoisomerase IIα. Rabbit anti-topoisomerase IIα primary antibody (ab12318) was from AbCam. Donkey antirabbit secondary antibody (IRDye 680 LT; 926-68023) was from Odyssey. Immunoblots were visualized and quantified using a Li-Cor Odyssey system.

RESULTS AND DISCUSSION

Effects of CP-115,955-Based Compounds on DNA Cleavage Mediated by Wild-type and Quinolone-Resistant GrlA^{S81F} and GrlA^{S81Y} Topoisomerase IV from *B. anthracis*. CP-115,955 is an experimental quinolone that differs from ciprofloxacin only by the substitution of a 4-hydroxyphenyl group for the C7 piperazinyl moiety. This drug (as well as CP-115,953, which differs from CP-115,955 by the inclusion of a fluorine at C8) has high activity against both bacterial and human type II topoisomerases.[19,32,38,39] Quinolones and quinazolinediones that contain a 3′-(AM)P group at C7 display a similar cross-reactivity and do so by forming novel interactions between the 3′-(AM)P group and the enzyme-cleaved DNA complex.[32] To determine whether the C7 4-hydroxyphenyl group also establishes novel contacts within the bacterial cleavage complex, the effects of a CP-115,955-based series of quinolones, quinazolinediones, and non-amino-quinazolinediones on DNA cleavage mediated by wild-type and quinolone-resistant GrlA^{S81F} and GrlA^{S81Y} *B. anthracis* topoisomerase IV were examined (Figure 2).

CP-115,955 showed high activity against wild-type *B. anthracis* topoisomerase IV (Figure 2A). However, the GrlA^{S81F} and GrlA^{S81Y} enzymes (which contain mutations in the residues

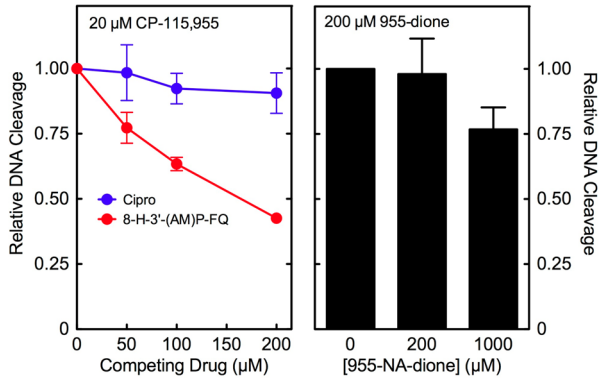

Figure 5. Contribution of quinolone and quinazolinedione substituents to drug interactions in the human topoisomerase IIα-DNA cleavage complex. The ability of ciprofloxacin (blue) and 8-H-3′-(AM)P-FQ (red) to compete out cleavage induced by 20 μM CP-115,955 is shown at the left. The ability of 955-NA-dione to compete out cleavage induced by 200 μM 955-dione is shown at the right. Drugs were added to reactions simultaneously. Initial levels of DNA cleavage (i.e., in the absence of competitor) were set to 1 to facilitate comparisons. Error bars represent the standard deviation of at least three independent experiments.

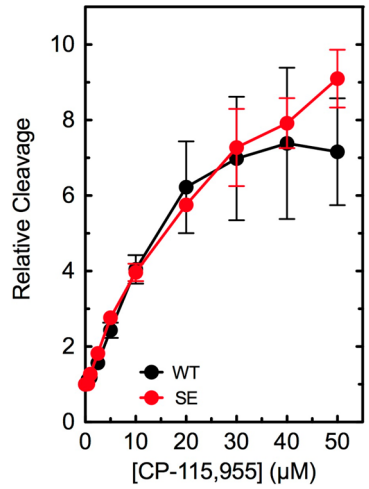

Figure 6. DNA cleavage induced by CP-115,955 with wild-type (black) and M762S/M766E mutant human topoisomerase IIα (red). Error bars represent the standard deviation of at least three independent experiments. The baseline level of DNA cleavage activity mediated by the mutant enzyme is ~3−fold lower than observed with wild-type topoisomerase IIα.[32] Therefore, results are plotted as "Relative DNA Cleavage" to allow a direct comparison between enzymes.

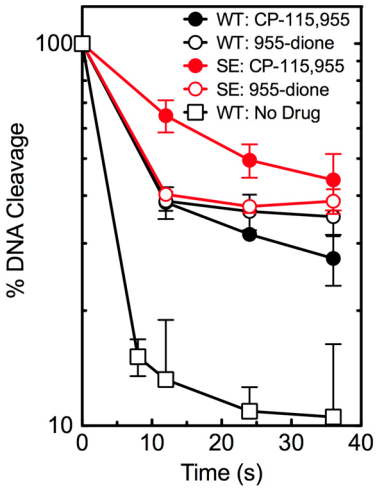

Figure 7. Effects of CP-115,955 and 955-dione on the persistence of ternary enzyme−drug−DNA cleavage complexes formed with wild-type and M762S/M766E mutant human topoisomerase IIα. The stability of wild-type (black circles) and hTop2A$^{M762S/M766E}$ (red circles) cleavage complexes formed in the presence of 20 μM CP-115,955 is shown. The stability of wild-type (open black circles) and hTop2A$^{M762S/M766E}$ (open red circles) cleavage complexes formed in the presence of 200 μM 955-dione is shown for comparison. The stability of wild-type cleavage complexes formed in the absence of drugs (open black squares) also is shown. Cleavage at time zero was set to 100% to facilitate direct comparisons. Error bars represent the standard deviation of at least three independent experiments.

that anchor the water−metal ion bridge) were resistant to the quinolone. This finding suggests that when bridge function is impaired, CP-115,955 shows little ability to enhance DNA cleavage mediated by *B. anthracis* topoisomerase IV. To further address the contribution of the C7 4-hydroxyphenyl group to the activity of CP-115,955, the activities of parallel compounds built on the quinazolinedione and non-amino-quinazolinedione scaffolds were assessed. Neither of these scaffolds are able to

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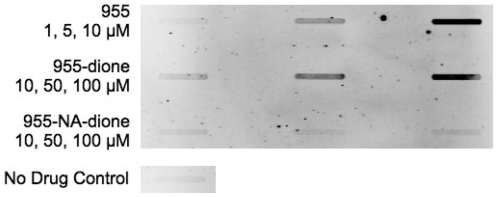

Figure 8. Drug-induced DNA cleavage mediated by topoisomerase IIα in cultured human CEM cells. The ability of CP-115,955, 955-dione, and 955-NA-dione to induce DNA cleavage by topoisomerase IIα was determined using the RADAR assay following a 1 h treatment. An immunoblot is shown and is representative of three independent experiments. The no drug control was from a different portion of the same blot.

support the formation of the water−metal ion bridge.[19,20,32] As seen in Figure 2D,G, the wild-type and mutant topoisomerase IV enzymes all displayed resistance to these compounds. Thus, we conclude that CP-115,955 interacts with the bacterial type II topoisomerase primarily through the water−metal ion bridge rather than the C7 4-hydroxyphenyl moiety.

To further assess interactions between CP-115,955-based drugs and topoisomerase IV, a series of C7 4-hydroxyphenyl- quinolones, -quinazolinediones, and -non-amino-quinazolinediones containing a methyl or methoxy group at C8 were examined (Figure 2B,C,E,F,H,I). Generally, the presence of a group at C8 enhanced drug activity against both the wild-type and mutant enzymes. In the absence of the N3 amino group, quinazolinediones that contain a C7 4-hydroxyphenyl ring displayed no ability to increase topoisomerase IV-mediated DNA cleavage. This suggests that additional interactions generated by the moiety at C8 are not strong enough to compensate for the loss of the N3 amino group. It is notable that similar trends have been reported for quinolones and quinazolinediones that contain clinically relevant C7 groups that do not contribute significantly to drug binding.[32]

Docking studies were carried out to further explore interactions between the C7 4-hydroxyphenyl ring of CP-115,955 and topoisomerase IV. Computer models were based on the structure of moxifloxacin in a cleavage complex formed with *A. baumannii* topoisomerase IV. Figure 3 compares the structure of the moxifloxacin complex with the modeled complex of 8-methoxy-955. These two quinolones differ only by the substitution of the C7 4-hydroxyphenyl ring of 8-methoxy-955 in place of the diazabicyclononyl ring of moxifloxacin.

Moxifloxacin is anchored to helix-4 via the water−metal ion bridge and an amine in the C7 ring interacts with dA20 (Figure 3, left). When the cognate CP-115,955 analog (8-methoxy-955) was modeled into this structure in place of moxifloxacin (Figure 3, right), the quinolone was still anchored to the protein via the water−metal ion bridge. However, there are no available binding interactions for the C7 phenol group. A previous modeling study indicates that quinolones and quinazolinediones containing a C7 3′-(AM)P group overcome resistance by forming an interaction between the C7 group and Glu437, a residue that is conserved among the bacterial type II topoisomerases.[52] This interaction was not observed for 8-methoxy-955. Thus, docking studies support the conclusion that quinolones in the CP-115,955 series interact with bacterial topoisomerase IV primarily through the water−metal ion bridge rather than the C7 moiety.

Effects of CP-115,955-Based Compounds on DNA Cleavage Mediated by Human Topoisomerase IIα. To explore the interactions between CP-115,955-based compounds and human type II topoisomerases, we first tested CP-115,955, 8-methyl-955, and 8-methoxy-955 for their ability to enhance DNA cleavage mediated by wild-type topoisomerase IIα (Figure 4). The level of double-stranded DNA cleavage induced by the anticancer drug etoposide is shown for comparison.

All three quinolones displayed high activity against the human enzyme. This result is in contrast to parallel quinolones that contained a 3′-(AM)P group at C7. These latter compounds required a substituent at C8 in order to induce enzyme-mediated DNA cleavage.[32] As determined by competition assays, the C8 substituent did not contribute to drug binding but was required to properly align the quinolone to stabilize DNA cleavage complexes.[32] The fact that the CP-115,955 series did not require a C8 substituent for potent activity implies that the C7 4-hydroxyphenyl moiety mediates both drug binding and alignment.

Next, we examined the ability of the parallel series of CP-115,955-based quinazolinediones and non-amino-quinazolinediones to induce DNA cleavage mediated by topoisomerase IIα (Figure 4). In all cases, the activity of the quinazolinedione was substantially lower than that of the corresponding quinolone. Furthermore, no activity was seen with any of the non-amino-quinazolinediones. These findings are despite the fact that the human type II enzyme cannot support the water−metal ion bridge. Thus, there appears to be an intrinsic advantage to the quinolone core, although the basis for this advantage is not known at the present time. Moreover, if a quinazolinedione core is employed as the drug scaffold, the N3 amino group appears to be critical for activity. Similar conclusions have been drawn for drugs containing a C7 3′-(AM)P group.[32]

Role of Drug Substituents in Mediating the Activity of CP-115,955 Against Human Topoisomerase IIα. A series of competition experiments was carried out to define how substituents on CP-115,955 contributed to the activity of the quinolone against topoisomerase IIα. In the first experiment, the contribution of the C7 4-hydroxyphenyl moiety to the binding of CP-115,955 in the topoisomerase IIα-cleaved DNA complex was examined by assessing the ability of ciprofloxacin to compete against 20 μM CP-115,955. Ciprofloxacin differs from CP-115,955 only by the presence of a C7 piperazinyl group in place of the 4-hydroxyphenyl moiety. Although the antibacterial quinolone displays virtually no ability to enhance DNA cleavage mediated by the human type II enzyme, it can compete with etoposide.[54] This finding demonstrates that ciprofloxacin shares an interaction domain with topoisomerase II poisons. As seen in Figure 5 (left), ciprofloxacin competed very poorly with CP-115,955. Cleavage was reduced only 10% at a 10-fold molar excess of ciprofloxacin over CP-115,955. This lack of competition provides strong evidence that the hydroxyphenyl moiety of CP-115,955 makes a major contribution to drug binding.

In the second experiment, the relative affinities of the C7 4-hydroxyphenyl and 3′-(AM)P groups were compared. To this point, a previous study examined the ability of ciprofloxacin to compete with 8-methyl-3′-(AM)P-FQ.[32] Topoisomerase IIα-mediated DNA cleavage induced by 8-methyl-3′-(AM)P-FQ was reduced ∼18% and ∼63% at a 1:1 ratio and a 10-fold molar excess of ciprofloxacin, respectively. When compared to the above result, this finding suggests that the C7 4-hydroxyphenyl

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moiety mediates tighter binding than does the C7 3′-(AM)P with the human system. To determine the relative affinities of these two C7 groups more directly, we examined the ability of 8-H-3′-(AM)P-FQ to compete with 20 μM CP-115,955. 8-H-3′-(AM)P-FQ displayed little effect on DNA cleavage mediated by topoisomerase IIα up to 200 μM drug.[32] As seen in Figure 5 (left), a 7.5-fold molar excess of 8-H-3′-(AM)P-FQ was required to reduce CP-115,955-induced DNA cleavage ∼50%. Thus, the hydroxyphenyl moiety appears to interact more strongly with human topoisomerase IIα than does the 3′-(AM)P group.

In the third experiment, the contribution of the N3 amino group of the quinazolinedione core to drug binding was assessed by characterizing the ability of 955-NA-dione to compete with 200 μM 955-dione. As shown above (Figure 4), removing the N3 amino group abrogated drug activity for all of the quinazolinediones that contained a C7 4-hydroxyphenyl group. Given the high concentration of 955-dione required to induce activity, competition could only be carried out to a 5:1 ratio. Even at a 5-fold excess of 955-NA-dione, cleavage was reduced by only ∼23% (Figure 5, right). This finding indicates that the N3 amino group contributes to the binding of the quinazolinedione core to the human enzyme−DNA complex.

Fluoroquinolones containing a 3′-(AM)P group at the C7 position display high activity against wild-type and quinolone-resistant *B. anthracis* topoisomerase IV due to strong interactions with the C7 group.[32] Consequently, drug activity is not further enhanced by the presence of the water−metal ion bridge.[32] Because the strong interaction between the human enzyme and the hydroxyphenyl ring of CP-115,955 parallels the above circumstance, we determined whether the introduction of bridge-anchoring residues in topoisomerase IIα would further enhance the activity of the quinolone. Mutation of Met762 and Met766 of human topoisomerase IIα to serine and glutamic acid, respectively (the residues seen in *B. anthracis* topoisomerase IV that coordinate the water-metal ion bridge), sensitizes the human enzyme to clinically relevant quinolones that do not interact with topoisomerase IIα through their C7 groups.[32] In contrast, the levels of cleavage induced by CP-115,955 in the presence of wild-type and mutant topoisomerase IIα were indistinguishable (Figure 6). Therefore, it appears that the strong interaction with the C7 4-hydroxyphenyl group of CP-115,955 precludes the necessity for a second interaction.

An alternative explanation is that CP-115,955 is unable to utilize the water−metal ion bridge in the human enzyme even in the presence of the anchoring residues. To distinguish these two possibilities, we examined the persistence of cleavage complexes formed by wild-type or hTop2A$^{M762S/M766E}$ in the presence of 20 μM CP-115,955 or 200 μM 955-dione (as a control drug that cannot form the water-metal ion bridge). 955-dione was used at a higher concentration than the quinolone to generate similar levels of cleavage complexes as a starting point. With wild-type topoisomerase IIα, drug-induced cleavage complexes were ∼2.5-fold more stable than those formed in the absence of drugs (Figure 7). Furthermore, hTop2A$^{M762S/M766E}$ cleavage complexes formed in the presence of CP-115,955 were ∼3-fold more stable than those formed by the wild-type enzyme in the presence of this drug, while there was no difference in the persistence of cleavage complexes formed by the two enzymes in the presence of 955-dione (Figure 7). This finding suggests that CP-115,955 can utilize the water−metal ion bridge in the presence of the anchoring residues. Furthermore, although the presence of the bridge interaction stabilizes CP-115,955-induced DNA cleavage complexes ∼3-fold, maximal levels of enzyme-mediated DNA cleavage activity can be achieved in its absence.

Effects of CP-115,955-Based Compounds on Levels of Topoisomerase IIα-DNA Cleavage Complexes Formed in Cultured Human CEM Cells. In order to determine whether CP-115,955-based compounds induce the formation of topoisomerase IIα-DNA cleavage complexes in human cells, CP-115,955, 955-dione, and 955-NA-dione were tested in CEM cells using the RADAR assay.[55] In this assay, cells are treated with compounds for 1 h, and cleavage complexes are trapped by rapid lysis in the presence of an anionic detergent. DNA is then isolated by ethanol precipitation, and the amount of topoisomerase IIα that is covalently attached to DNA is measured by immunoblotting (Figure 8). It is notable that all of the drugs tested displayed some insolubility in the tissue culture medium; therefore, results should only be interpreted in terms of relative effects.

CP-115,955 increased levels of topoisomerase IIα cleavage complexes at less than 10 μM drug. Although 955-dione induced little cleavage at 10 μM, it enhanced DNA scission at 50−100 μM drug. In contrast, 955-NA-dione showed no activity at any concentration tested. The relative activities of these compounds in cells reflected their abilities to induce enzyme-mediated DNA cleavage in *in vitro* cleavage assays.

Summary. CP-115,955 is a quinolone with a 4-hydroxyphenyl at C7 that displays high activity against both *B. anthracis* topoisomerase IV and human topoisomerase IIα. However, the drug uses different structural features to recognize the two enzymes. Although the C7 4-hydroxyphenyl group establishes strong contacts with topoisomerase IIα, it does not mediate interactions with topoisomerase IV. Rather, as seen with clinically relevant quinolones, CP-115,955 interacts with bacterial type II topoisomerases primarily through the water−metal ion bridge. Therefore, this C7 group can distinguish between the bacterial and human type II topoisomerases. This is in contrast to quinolones with C7 3′-(AM)P groups, which maintain high activity against the bacterial and human enzymes by establishing interactions with both through the C7 moiety. Quinolones show higher activity than quinazolinediones against topoisomerase IIα *in vitro* and in cultured cells. In addition, compounds with a C7 4-hydroxyphenyl group (which is found in the pendant rings of several topoisomerase II-active compounds[56−58]) are stronger topoisomerase II poisons than comparable compounds containing a 3′-(AM)P group. These findings suggest that quinolones in the CP-115,955 series could be a suitable starting point for the development of topoisomerase II-targeted drugs with anticancer potential.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Table S1 contains the full chemical, library, and abbreviated names of the compounds used in this study. Methods of preparation and structural characterization of compounds synthesized for this study also are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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