DNA Gyrase Interaction with Coumarin-Based Inhibitors: The Role of the Hydroxybenzoate Isopentenyl Moiety and the 5′-Methyl Group of the Noviose†

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ABSTRACT: DNA gyrase is a major bacterial protein that is involved in replication and transcription and catalyzes the negative supercoiling of bacterial circular DNA. DNA gyrase is a known target for antibacterial agents since its blocking induces bacterial death. Quinolones, coumarins, and cyclothialidines have been designed to inhibit gyrase. Significant improvements can still be envisioned for a better coumarin−gyrase interaction. In this work, we obtained the crystal costructures of the natural coumarin clorobiocin and a synthetic analogue with the 24 kDa gyrase fragment. We used isothermal titration microcalorimetry and differential scanning calorimetry to obtain the thermodynamic parameters representative of the molecular interactions occurring during the binding process between coumarins and the 24 kDa gyrase fragment. We provide the first experimental evidence that clorobiocin binds gyrase with a stronger affinity than novobiocin. We also demonstrate the crucial role of both the hydroxybenzoate isopentenyl moiety and the 5′-alkyl group on the noviose of the coumarins in the binding affinity for gyrase.

DNA gyrase is a bacterial protein of the topoisomerase family involved in DNA replication and transcription (for a review see refs *1* and *2*). The *Escherichia coli* DNA gyrase is a type II topoisomerase catalyzing the negative supercoiling of the closed-circular DNA using the free energy released by ATP hydrolysis (*3*, *4*). As this function is essential for DNA replication and transcription, gyrase is really a suitable target for antibacterial agents (*5*). The DNA gyrase of *E. coli* is composed of two subunits, A (97 kDa) and B (90 kDa), forming an A_2B_2 tetramer (*6*−*8*). The A subunit is mainly involved in DNA breakage and reunion, while the B subunit exhibits ATPase activity (*3*, *4*). The B subunit consists of two domains, an N-terminal domain of ∼44 kDa and a C-terminal one of 47 kDa (*9*). The N-terminal domain includes two subdomains (24 kDa C-terminal part and 20 kDa N-terminal part). The ATP-binding site is located in the first subdomain, i.e., in the C-terminal part of the N-terminal domain (*10*).

Over the years, several classes of antibiotics have been used as inhibitors of gyrase activity (for a review, see ref *5*). Three have shown high efficiency: quinolones (e.g., norfloxacin) (*11*), coumarins (*12*, *13*), such as novobiocin and clorobiocin (Figure 1), and cyclothialidines (*14*). Quinolones interact with subunit A of DNA gyrase (*11*), while cyclothialidines and coumarins interact with subunit B of

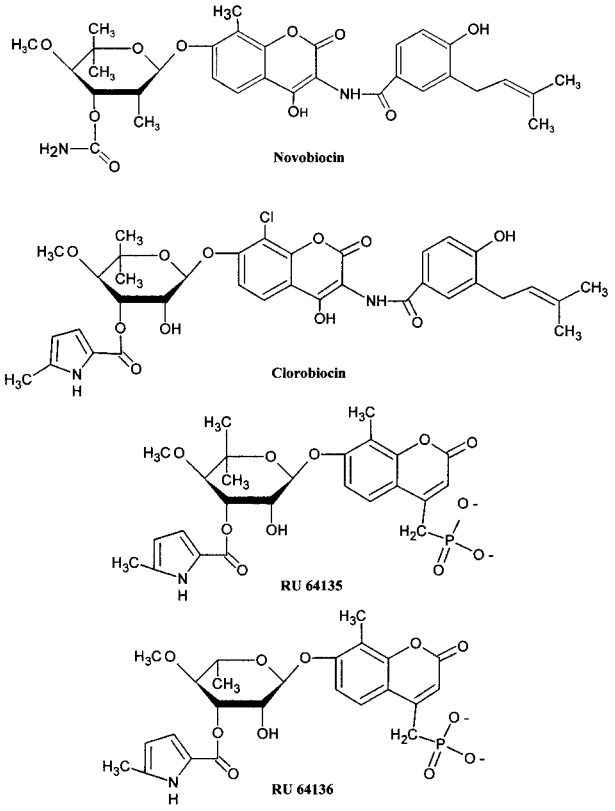

FIGURE 1: Structures of novobiocin, clorobiocin, and two synthetic coumarin analogues, RU 64135 and RU 64136.

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the protein (*15*). Clorobiocin and novobiocin are the most important members of the coumarin family. These drugs are natural compounds inhibiting gyrase action by competitively binding to the ATP-binding site even if the coumarin binding site does not totally overlap the ATP-binding site (*15*) as previously shown by the crystal structures of the 24 kDa gyrase domain−inhibitor complexes (*13*−*15*). It has also been shown that the binding properties of coumarinic ligands toward the 24 kDa fragment are rather similar to those of the entire B subunit (*16*, *17*).

Since both classes of antibiotics have limitations and side effects (such as jaundice), the search for new gyrase inhibitors is still of prime importance. Numerous synthetic coumarins with limited side effects have been designed (*18*, *19*); among them, RU 64135 and RU 64136 are of particular interest (Figure 1) (*20*).

The aims of our work were (1) to determine the accurate binding parameters for clorobiocin (Figure 1) and compare them with those of the leading compound, novobiocin (Figure 1), and (2) to estimate the contribution of the hydroxybenzoate isopentenyl group and of the 5′-methyl one of the noviose to the clorobiocin affinity for gyrase. For this purpose, we synthesized coumarins lacking the hydroxybenzoate isopentenyl moiety (RU 64135 and RU 64136) and the 5′-methyl group on the noviose (RU 64136) (Figure 1). The crystal structure of the clorobiocin−24 kDa gyrase complex has been reported, but the coordinates have not been deposited in the Protein Data Bank (*14*). We determined the X-ray structure of the clorobiocin− and RU 64135−24 kDa gyrase fragment complexes. We applied isothermal titration microcalorimetry (ITC)[1] and differential scanning calorimetry (DSC) to obtain the thermodynamic parameters characteristic of the molecular interactions occurring during the binding process between the three coumarins, clorobiocin, RU 64135, and RU 64136, and the 24 kDa gyrase fragment. We provide for the first time the binding affinity for clorobiocin obtained by a displacement method, and our analysis of the thermodynamic parameters, in line with the X-ray structures, highlights the crucial role of the isopentenyl moiety and the 5′-methyl group of the noviose for the interaction with gyrase. This study allows a better understanding of the key contacts between the coumarins and the enzyme which are especially important for a rational drug design program.

EXPERIMENTAL PROCEDURES

Protein and Antibiotics. The 24 kDa fragment of *E. coli* DNA gyrase was produced and purified as described previously (*16*). A stock solution (84 mg/mL in Tris buffer, 150 mM NaCl, and 5% glycerol) was aliquoted and stored at −80 °C. Electrospray ionization mass spectrometry confirmed the protein purity. Prior to each experiment, DNA gyrase was dialyzed overnight against experimental buffer [20 mM Tris and 1 mM EDTA (pH 7.5) or 20 mM phosphate and 1 mM EDTA (pH 7.5)]. Protein concentrations were determined using amino acid analysis or UV absorption at 280 nm. Clorobiocin, RU 64135, and RU 64136 were obtained and purified as described previously (*20*). Clorobiocin was dissolved in 100% methanol and then diluted with the buffer to ensure that the final concentration of methanol was 3%; for the corresponding ITC experiments, 3% methanol was added to the protein solution. RU 64135 and RU 64136 were dissolved in the protein dialysis buffer.

Isothermal Titration Calorimetry. Binding of gyrase to coumarin analogues was carried out at 25 °C and pH 7.5 in 1 mM EDTA, 20 mM Tris, or 20 mM phosphate, using a MicroCal MCS titration calorimeter. The enthalpy of binding (ΔH), affinity constant (K_a), and molar binding stoichiometry were obtained as follows. Aliquots (5−10 μL) of ligands ($2-4 \times 10^{-4}$ M) were injected from a 250 μL microsyringe into the 1.34 mL calorimeter cell containing the protein solution ($1.0-3.5 \times 10^{-5}$ M) to achieve a complete binding isotherm. The heat of dilution was measured by injecting the ligand into the buffer solution or by additional injections of ligand after saturation; the value that was obtained was subtracted from the heat of reaction to obtain the effective heat of binding. Titration curves were fitted using MicroCal Origin software, assuming one set of sites. Changes in free energy (ΔG) and entropy (ΔS) were calculated from the relationship $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$.

When the constant of association was too high to be measured directly, we used a method called "displacement titration calorimetry" (*21*, *22*) that includes the measurement of an apparent binding constant (K_{app}) corresponding to the displacement of a low-affinity ligand by a stronger one. In this case, the 24 kDa gyrase fragment was saturated with a low-affinity ligand L_1 (RU 64136) introduced into the calorimeter cell and then titrated with 10 μL aliquots of high-affinity ligand L_2 (clorobiocin). An apparent binding constant, corresponding to the displacement of L_1 by L_2, was thus obtained. Then, knowing the binding constant K_1 of L_1, we obtained the value of the binding constant K_2 of L_2 for gyrase from the equation $K_2 = K_{app}(1 + K_1[L_1])$.

The reported values of K_a and ΔH are the means from at least five measurements with the standard deviation.

The change in heat capacity of binding (ΔC_p) was obtained by measuring the change of the binding enthalpy over a range of temperatures from 13 to 30 °C from the relationship $\Delta C_p = d(\Delta H)/dT$, assuming that ΔH approximates to a linear function of temperature.

Differential Scanning Microcalorimetry (DSC). Heat denaturation measurements were carried out on a MicroCal VP-DSC instrument in 0.51 mL cells at a heating rate of 1 K/min. Protein concentrations varied from 10 to 30 μM. Saturated complexes of the 24 kDa gyrase fragment with ligands in 20 mM Tris and 1 mM EDTA (pH 7.5) were taken and analyzed immediately after ITC experiments. Curves were corrected for the instrumental baseline obtained by heating the solvent used for protein solution. The reversibility of denaturation was checked routinely by sample reheating after cooling in the calorimetric cell. The partial molar heat capacity of the protein (C_p) and denaturation temperature (T_d) were determined as described elsewhere (*23*), with the partial specific volume of 0.74 cm^3/g calculated according to ref *24*. To analyze functions of excess heat capacity, MicroCal Origin software was used. The accuracy of the denaturation temperature was within 0.5 °C.

Circular Dichroism (CD). Near-UV CD spectra were registered with a Jasco J-715 spectropolarimeter equipped with Neslab RTE-111 water bath. The cell had a light path of 10 mm; protein samples were the same as for DSC. Melting experiments were performed at a heating rate of

[1] Abbreviations: ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; CD, circular dichroism.

Table 1: Data Collection and Processing Statistics for the 24 kDa Gyrase Fragment−Inhibitor Complexes

complex	space group	data processing
24 kDa gyrase−clorobiocin	$P2_12_12_1$ $a = 40.71$ Å, $b = 47.63$ Å, $c = 111.60$ Å diffraction limit, 30−2 Å	16938 unique reflections completeness = 85.0% $R_{merge} = 4.25\%$ redundancy = 2.86
24 kDa gyrase−RU 64135	$P2_12_12_1$ $a = 46.19$ Å, $b = 60.75$ Å, $c = 64.82$ Å diffraction limit, 28.5−1.8 Å	17209 unique reflections completeness = 98.4% $R_{merge} = 3.0\%$ redundancy = 4.6

1 K/min. Denaturation temperatures were determined from the peaks in the first derivative of the melting profile (accuracy of 0.5 °C).

Crystallization and Structure Determination of the 24 kDa Gyrase Fragment−Clorobiocin and 24 kDa Gyrase Fragment−RU 64135 Complexes. All crystallization trials were carried out in Linbro 24-well tissue-culture plates (Hampton Research) using the hanging-drop vapor-diffusion method. The 24 kDa gyrase fragment concentrated to 20 mg/mL containing 0.5 mM inhibitor was mixed with an equal volume of crystallization buffer containing 100 mM Hepes (pH 7.5), 24% PEG 20000, 12% propanol-2 equilibrated against a well containing the same buffer at 297 K. Due to the cryoprotectant character of the crystallization medium, crystals could be directly flash-frozen in liquid nitrogen. Diffraction data were measured at 140 K from a single crystal using synchrotron radiation at LURE (Orsay, France). For the 24 kDa gyrase fragment−RU 64135 complex, data were collected in two passes (low and high resolution) to optimize the signal-to-noise ratio. Reflections were recorded using a MAR image plate detector, processed with DENZO, and scaled together with SCALEPACK (*25*). Crystals of both complexes belong to the $P2_12_12_1$ space group with one molecule of the complex per asymmetric unit (see Table 1 for statistics). A molecular replacement solution was obtained using AMORE (*26*) with the structure of the corresponding fragment complexed with novobiocin (*15*) as a search model. The solvent-flattened map was of sufficient quality to fit the inhibitors in the electron density. The molecular models were refined using CNS (*27*) interspersed with model building in O6 (*28*). After manual rebuilding, all but 20 residues of the loop of residues 98−118 could be fitted. The loop itself is disordered in the crystal. The final model of the 24 kDa gyrase domain−clorobiocin complex consists of residues 15−219 [except the residues from the disordered loop (98−118)], the clorobiocin, and 200 water molecules with an R factor of 23% and an R_{free} of 28% (using 5% of the reflections). The final model of the 24 kDa gyrase domain−RU 64135 complex consists of residues 11−218 [except the residues from the disordered loop (98−118)], the inhibitor, and 139 water molecules with an R factor of 22% and an R_{free} of 27% (using 5% of the reflections).

RESULTS

Clorobiocin, RU 64135, and RU 64136 Binding to the 24 kDa Gyrase Fragment. Binding of the synthetic analogues RU 64135 and RU 64136 to the 24 kDa fragment of DNA gyrase was studied by ITC. A typical set of data for the RU 64135 binding to the 24 kDa gyrase fragment is shown in Figure 2; panel A presents the raw calorimetric data for the ligand-into-protein titration and panel B the binding isotherm.

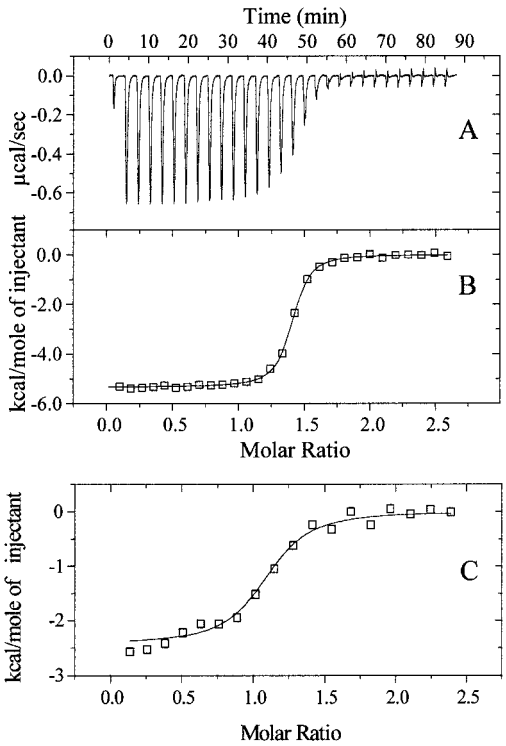

FIGURE 2: (A) ITC titration of the 24 kDa gyrase fragment (3.6×10^{-5} M) with RU 64135 (4.0×10^{-4} M) at 25 °C in 20 mM Tris and 1 mM EDTA (pH 7.5). (B) Binding isotherm derived from panel A. (C) Binding isotherm for the titration by clorobiocin (3×10^{-4} M) of the 24 kDa gyrase fragment (1.5×10^{-5} M) initially saturated with RU 64136 (4×10^{-4} M).

The affinity of clorobiocin for gyrase is too strong to be accurately determined by direct ITC measurements (*13*). Indeed, one of the ITC limitations is the determination of high association constants, and for the vast majority of protein−ligand interactions, constants higher than 10^8 M^{-1} cannot be measured since the low protein concentration required for such tight binding reactions will be associated with an excessively low level of heat exchange. Thus, we measured the enthalpy and stoichiometry for clorobiocin binding by a standard ITC method, while the association constant was determined by the displacement method (*21*, *22*); i.e., gyrase was saturated with RU 64136 which was further displaced with clorobiocin (Figure 2C). Parameters for binding of these three compounds to the 24 kDa fragment of DNA gyrase at pH 7.5 are given in Table 2.

Using the displacement method, the affinity of clorobiocin for 24 kDa gyrase fragment was accurately determined for the first time [$K_a = (8.6 \pm 2.2) \times 10^8$ M^{-1} (Table 2)]. This K_a value is at least 15 times higher than the previously published values obtained by direct ITC measurements ($2-6 \times 10^7$ M^{-1}) (*12*, *14*) and ~20 times higher than those of

Table 2: Thermodynamic Parameters of the Coumarin Analogues Binding to the 24 kDa Fragment of DNA Gyrase in 20 mM Tris and 1 mM EDTA (pH 7.5)

	K_a (M^{-1})	ΔH (kJ/mol)	ΔG (kJ/mol)	$T\Delta S$ (kJ/mol)
clorobiocin	$(8.6 \pm 2.2) \times 10^8$ [a]	-39.6 ± 2.4	-51.0 ± 0.6	11.4 ± 1.0
RU 64135	$(1.2 \pm 0.2) \times 10^7$	-18.9 ± 3.1	-40.3 ± 0.5	21.4 ± 1.6
RU 64136	$(1.4 \pm 0.1) \times 10^6$	-18.0 ± 0.5	-35.1 ± 0.2	17.1 ± 0.3
novobiocin[b]	$(2.8 \pm 0.8) \times 10^7$	-51.8 ± 4.2	-42.7 ± 0.5	-9.1 ± 3.9

[a] Displacement titration measurements. [b] Mean values of refs *12*−*14*.

novobiocin (*12*−*14*) (Table 2). Affinity constants of RU 64135 and RU 64136 are equal to $(1.2 \pm 0.2) \times 10^7$ and $(1.4 \pm 0.1) \times 10^6$ M^{-1}, respectively (Table 2). The stoichiometry of the interaction is close to unity in all cases, indicating the 1:1 complex formation.

Clorobiocin, RU 64135, and RU 64136 binding to gyrase is both enthalpically and entropically favored (Table 2). However, striking differences exist between the three compounds since the absolute enthalpy values for both RUs are twice lower than those for clorobiocin, whereas entropy values are more favorable in the case of RUs than in the case of clorobiocin (Table 2). To examine the contribution of the heat of buffer ionization to complex formation, experiments were performed in Tris and phosphate buffers. Despite the different heats of ionization for these buffers (47.2 kJ/mol for Tris and 3.3 kJ/mol for phosphate) (*29*), the measured enthalpy of binding is practically independent of buffer. Thus, the net flux of protons for the interaction of the three drugs with the 24 kDa gyrase fragment must be around zero.

Heat Capacity of Binding. The change in the heat capacity of binding (ΔC_p) is related to solvation changes between two states of a system and can be determined by ITC measurements performed over a range of temperatures. This parameter is important for understanding how a protein adapts to the incorporation of the ligand. Conformational changes can expose or hide apolar surfaces, resulting in a positive (exposure) or negative (hiding) contribution to ΔC_p (*30*). In addition, the ligand binding decreases the protein surface accessibility to the solvent and hides apolar surfaces that are responsible for the major positive contribution to ΔC_p (*30*). The ITC measurements were carried out at four temperatures (13, 19.5, 25, and 30 °C) for all three ligands. The examination of thermal denaturation of the 24 kDa gyrase fragment (see below) shows that the unfolding starts just above 35 °C. Accordingly, we chose 30 °C as the highest temperature for performing the ITC measurements. The ΔH of binding was plotted against temperature (Figure 3A), and the data were fitted to a linear function to obtain the ΔC_p of binding. The ΔC_p values for the analyzed complexes are relatively high and negative; that of clorobiocin (-1.46 ± 0.14 kJ mol^{-1} K^{-1}) is considerably more negative than those for its synthetic analogues, and a small increase in the ΔC_p absolute value is noticed for the 24 kDa gyrase fragment−RU 64135 complex (-0.83 ± 0.07 kJ mol^{-1} K^{-1}) compared to that of the 24 kDa gyrase fragment−RU 64136 complex (-0.62 ± 0.06 kJ mol^{-1} K^{-1}).

Thermal Stability of the 24 kDa Gyrase Fragment and Its Coumarin Complexes. Temperature dependencies of the partial molar heat capacity of the 24 kDa gyrase fragment and its coumarin complexes exhibit two peaks; the reversibility is almost complete for the first peak, while the second transition is irreversible (a typical melting curve is shown in Figure 3B). Binding of the 24 kDa gyrase fragment to the ligands increases the temperatures of both peaks (Table 3). Denaturation of the gyrase−RU 64135 complex was also followed by changes in the CD amplitude at 296 nm (data not shown). As for DSC, two transitions were recorded whose temperatures practically coincide with those obtained by calorimetry (Table 3).

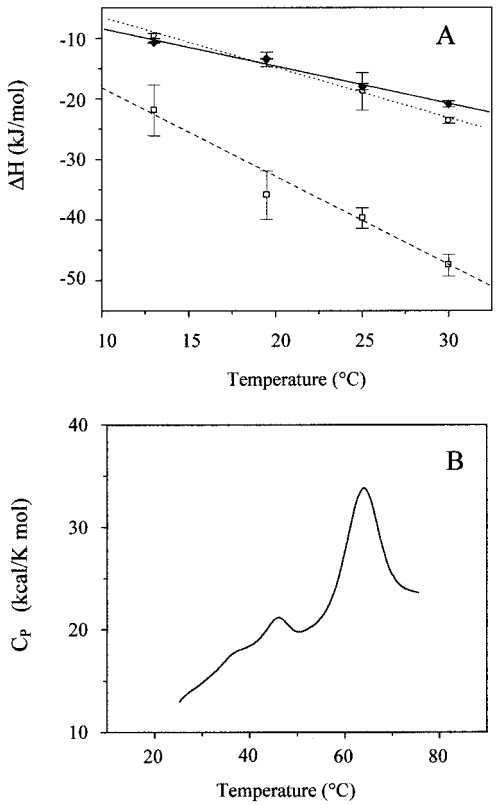

FIGURE 3: (A) Enthalpy of binding of RU 64135 (○), RU 64136 (◆), and clorobiocin (□) to the 24 kDa gyrase fragment as a function of temperature at pH 7.5. (B) Temperature dependence of the partial molar heat capacity of the 24 kDa gyrase fragment−RU 64135 complex in 20 mM Tris and 1 mM EDTA (pH 7.5).

Table 3: Parameters of Thermal Denaturation of the 24 kDa Fragment of DNA Gyrase and Its Complexes with Coumarin Analogues in 20 mM Tris and 1 mM EGTA (pH 7.5)

sample	first transition, T_1 (°C)	second transition, T_2 (°C)
gyrase	39.5	53.9
gyrase−clorobiocin	55.4	67.2
gyrase−RU 64135	46.9 (47.0[a])	64.4 (65.5[a])
gyrase−RU 64136	42.9	61.0

[a] Values of denaturation temperatures obtained from the CD change at 296 nm.

X-ray Structure of the 24 kDa Gyrase Fragment in Complex with Clorobiocin or RU 64135. The structures were

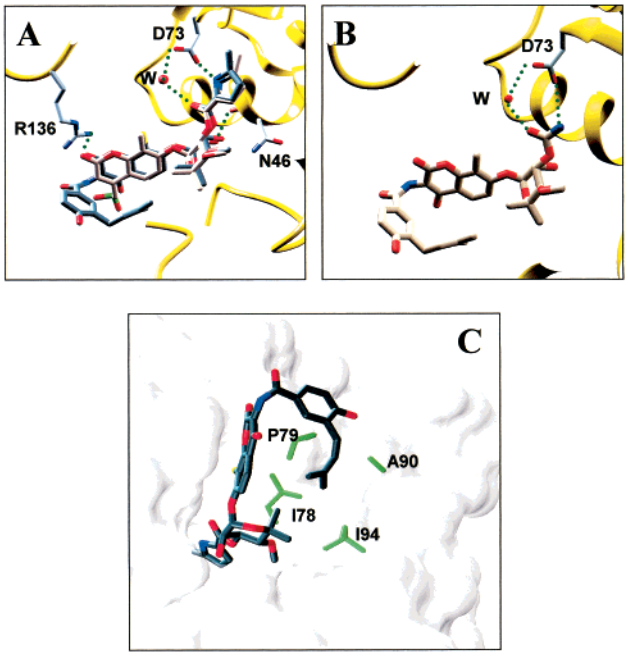

FIGURE 4: Positioning of the coumarins in the 24 kDa gyrase fragment binding site. (A) Superposition of the 24 kDa gyrase fragment−clorobiocin and −RU 64135 crystal structures showing that the two coumarins fill the same pocket with the conserved Arg136, Asp73, and Asn46 binding the inhibitors. (B) The novobiocin molecule is bound to the 24 kDa gyrase fragment by an H-bond network implicating Asp73 and a water molecule in a way similar to that of the pyrrole ring of clorobiocin and RU 64135. Two more water molecules participate in the binding of novobiocin (not shown). Coordinates of the novobiocin complex with the 24 kDa gyrase fragment were taken from PDB entry 1AJ6. (C) The hydroxybenzoate isopentenyl group of clorobiocin wraps around Pro79, making weak interactions with the hydrophobic surface (in pale yellow) also containing Ile78, Ala90, and Ala94 (in green).

determined by molecular replacement using the crystal structure of the 24 kDa gyrase fragment in complex with novobiocin determined by Holdgate et al. (*13*). As previously described for coumarins (*15*), the inhibitors lie at the entry of the catalytic pocket partially covering the ATP-binding site (Figure 4). In all structures, the loop closing the ATP-binding site between residues 98 and 118 is disordered as already observed with novobiocin and clorobiocin (*13*−*15*).

The most important contacts anchoring the coumarins in the 24 kDa gyrase domain are located in the region covering the ATP-binding site. The major interactions between DNA gyrase and RU 64135 are very similar to that observed with novobiocin (*17*). The main residues involved in inhibitor binding are the conserved Arg136 which interacts with the lactone coumarin ring and Asp46 that is in contact with a hydroxy group of the noviose (Figure 4A). Asp73 interacts via a water molecule with the terminal carboxy group of novobiocin, whereas it interacts with the carboxy pyrrole of RU 64135. In the complex with novobiocin (Figure 4B), the same residue also directly interacts with the terminal amino group, whereas it contacts the carboxy pyrrole nitrogen of both clorobiocin and RU 64135, thus maintaining a similar geometry of the H-bond network (Figure 4A,B). In contrast to novobiocin, the additional pyrrole ring of RU 64135 enters a large hydrophobic pocket. This pocket corresponds to the pyrrole pocket described in the previous structure of the 24 kDa gyrase fragment−clorobiocin complex (*14*) and consists of Val43, -47, -71, -120, and -167, Ala47, Ile78, and Met95.

RU 64135 is structurally similar to clorobiocin, bearing the coumarin ring coupled to the noviose sugar moiety and the methylpyrrole ring. The superposition of the 24 kDa gyrase fragment−clorobiocin complex and the 24 kDa gyrase fragment−RU 64135 complex indicates a similar positioning for both coumarins, noviose and pyrrole fragments. The main difference between RU 64135 and clorobiocin is the replacement of the hydroxybenzoate isopentyl group with the methyl phosphonate group. The hydroxybenzoate isopentyl group of clorobiocin lies outside the catalytic pocket, making weak interactions with a hydrophobic surface containing Ile78, Pro79, Ala90, and Ala94 (Figure 4C).

DISCUSSION

Until now, the existing thermodynamic data on the clorobiocin and novobiocin binding to gyrase revealed no difference between the two antibiotics (*12*−*14*). Hence, it was difficult to explain why clorobiocin is more active than novobiocin in vivo (*16*). The higher antibiotic activity of clorobiocin was linked to a better putative transmembrane permeability (*14*). On the other hand, X-ray studies indicated a major difference in the clorobiocin and novobiocin binding to gyrase; whereas three ordered water molecules are present in the novobiocin−gyrase complex, two of them, located in one of the hydrophobic pockets of the protein, are forced out by the pyrrole ring of clorobiocin (*13*, *14*). According to Gormley et al. (*13*), direct ITC measurements underestimated the binding constant and entropy change for the clorobiocin binding to gyrase (*14*), which was not the case for novobiocin. Using displacement titration calorimetry, we demonstrate a higher affinity of clorobiocin for the target gyrase enzyme.

Contrary to the novobiocin−gyrase complex, which is almost entirely enthalpically favored, our ITC measurements indicate that the interaction of clorobiocin with gyrase is both enthalpically and entropically favored (Table 2). The hydrogen and van der Waals bonding networks between clorobiocin and gyrase are responsible for the favorable enthalpy. The measured entropic effect corresponds to the balance between the constraining (negative ΔS) and the hydrophobic interactions (positive ΔS) (*31*). The positive value for ΔS of the system which we obtained (Table 2) indicates that the reorganization of water molecules of the solvent at the binding interface overpasses the effect of ligand constraining. The more favorable entropy in the case of clorobiocin binding compared to novobiocin (Table 2) proves, in agreement with the X-ray data, that the pyrrole ring of clorobiocin induces additional hydrophobic contacts, and water molecules present in the novobiocin−gyrase complex are forced out by the pyrrole ring in both clorobiocin and RU 64135 structures (Figure 5A). This action increases significantly the entropy of the system (from −9.11 to 11.4 kJ/mol), and the loss of some interactions with the ordered water molecules slightly decreases its enthalpy (Table 2). Therefore, the significant differences in the thermodynamic parameters between clorobiocin and novobiocin are well connected with water removal from the binding interface.

The superposition of the clorobiocin− and RU 64135−24 kDa gyrase fragment X-ray structures indicates a similar positioning of the coumarin, noviose, and methylpyrrole parts with similar binding modes and key amino acids residues

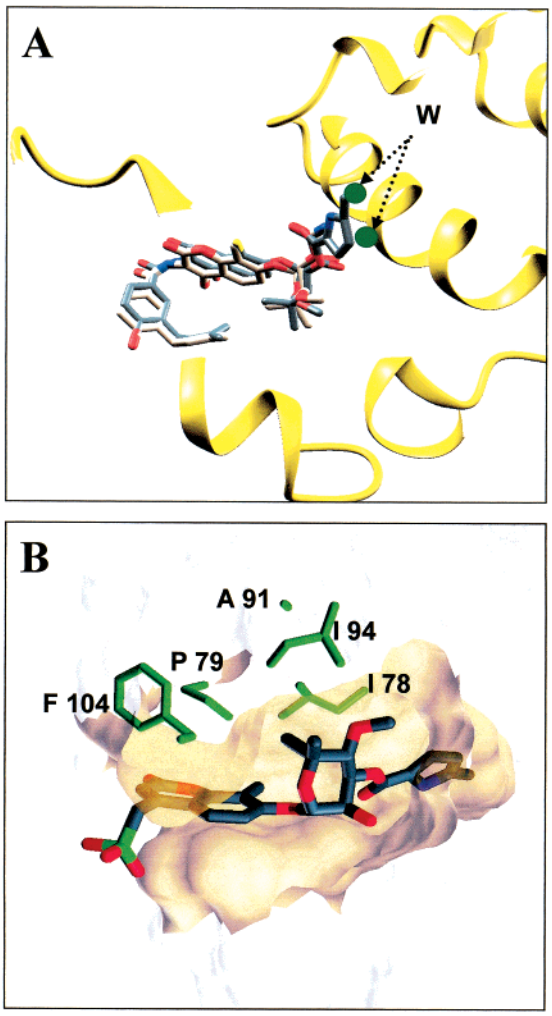

FIGURE 5: (A) Superposition of the 24 kDa gyrase fragment−clorobiocin and −novobiocin crystal structures showing that two water molecules (green dots) present with novobiocin in the pocket are excluded by the pyrrole ring of clorobiocin as previously observed (*14*). We observe the same water exclusion in the presence of the synthetic analogue RU 64135 also containing a pyrrole ring. (B) Section view of the 24 kDa gyrase fragment−RU 64135 catalytic pocket surface (in yellow with the protein overall surface in light gray). The dimethyl group of RU 64135 enters a small pocket consisting of five hydrophobic residues (in green).

involved in the interactions (Figure 4A). However, the hydroxybenzoate isopentenyl group in both RUs has been replaced with a methyl phosphonate (Figure 1). The isopentenyl group of clorobiocin wraps around Pro74 of gyrase and folds back away from the solvent onto the coumarin ring, reducing the overall hydrophobic surface of the antibiotic, as well as forming weak hydrophobic interactions with the protein (Figure 4C). Apart from the methyl group on the coumarin ring which is replaced with a chlorine, the rest of the RU 64135 molecule is identical to that of clorobiocin. According to our X-ray data, the methyl group on the coumarin ring of the RU 64135 like the chlorine of the clorobiocin does not make direct contacts with the 24 kDa gyrase fragment. Hence, in contrast with the previously published data (*14*), our ITC measurements reveal the important role of the isopentenyl group for the gyrase−coumarin affinity. Indeed, the binding constant of RU 64135 is 72 times lower than that of clorobiocin. This drastic loss in affinity correlates with the enthalpic loss (Table 2), thus pointing out that the level of hydrogen and/or van der Waals interactions between coumarins and gyrase is decreased, even if the superposition of clorobiocine and RU 64135 X-ray structures indicates a similar positioning of the coumarin, noviose, and methylpyrrole parts with similar binding modes and key amino acids residues involved in the interactions (Figure 4A). Major enthalpic changes indicate that the hydroxybenzoate isopentenyl group of clorobiocin plays a crucial role in the binding affinity, probably in the accommodation of clorobiocin into the binding site of gyrase, with subtle differences modifying the panel of polar and apolar interactions leading to an increased enthalpic gain. On the other hand, a more favorable entropic gain (21.4 vs 11.4 kJ/mol) is observed with RU 64135 versus clorobiocin, suggesting that RU 64135 is less constrained than clorobiocin upon binding to gyrase. Similar expulsion of two ordered water molecules is observed in both X-ray structures (Figure 5A).

The two RU compounds structurally differ only by a methyl group on the noviose sugar moiety (Figure 1). Note that the K_a of RU 64136 is 10 times lower than that of RU 64135, elucidating the major role of the noviose methyl group in the binding affinity. X-ray data indicate that this methyl group penetrates a gyrase smaller hydrophobic pocket, producing additional hydrophobic contacts involving residues from loops 98−118 and 78−86 (Figure 5B). The breakdown of these contacts is responsible for an entropic loss of 4.3 kJ/mol (whereas values of ΔH for RUs are the same). However, as one can see from the X-ray data, the methyl group on the noviose of RU 64135 (Figure 5B) does not occupy the whole hydrophobic cavity. Hence, an increased level of interaction resulting from an extended alkyl chain can be envisioned.

The two-peak melting curve of the 24 kDa fragment shows that it is composed of two different parts. The three ligands strongly stabilize both parts of the protein against thermal denaturation. The increase in protein stability goes along with the increase in protein affinity for the ligands. However, the general features of the thermal denaturation were the same for gyrase alone or gyrase complexed with coumarins; thus, we are confident that no drastic conformational changes appeared during the binding, and therefore, the ΔC_p of binding is connected with changes at the binding interface.

The negative ΔC_p value of novobiocin binding to gyrase (-1.23 kJ K^{-1} mol^{-1}) is attributed to hiding of hydrophobic residues at the binding interface (*13*), which is slightly compensated by trapping of water molecules [a trapped water molecule is responsible for a positive contribution to ΔC_p (*32*)]. The ΔC_p value for clorobiocin binding is more negative than the value for novobiocin binding. It can be explained by intrusion of the methylpyrrole moiety in a gyrase hydrophobic pocket and a subsequent decrease of the accessibility of apolar surfaces at the binding interface. RUs are characterized by a strikingly less negative ΔC_p than novobiocin and clorobiocin. This is linked to a better accessibility of hydrophobic patches due to the absence of the isopentenyl moiety.

To sum up, using the displacement titration calorimetry, we demonstrate that the higher affinity of clorobiocin is one of the parameters determining its higher anti-infective potency compared to novobiocin. We show that the presence of the hydroxylbenzoate isopentenyl moiety and the 5′-

methyl group of the noviose is responsible for a better interaction between the coumarins and the 24 kDa gyrase fragment and that the higher affinity correlates with a better temperature stabilization of the complex. Our data remove some of the existing doubts concerning the interaction between coumarins and the medically important enzyme gyrase. We can envision that new compounds with an extended alkyl chain at the 5′-position of the noviose and a conserved hydroxylbenzoate isopentenyl moiety will be more effective against bacterial infection. We are already using these data to screen new gyrase inhibitors with increased binding affinity.

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