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Antiviral 6-amino-quinolones: Molecular basis for potency and selectivity

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Abstract—Structural modifications introduced in 6-amino-quinolones to increase antiviral activity can strongly affect cytotoxicity to host cells. By competition to Tat–TAR complex and binding experiments to viral and cellular DNA and RNA structures, we show that the nature of the substituent at position 7 modifies drug affinity and specificity for the nucleic acid. Interestingly, the basicity of the above substituent modulates chelation of the quinolone template to magnesium ions, which, in turn, critically affects the potency and target selectivity in the antiviral quinolone family.

1. Introduction

Quinolones represent an important class of broad-spectrum antibacterials, whose main structural feature is a 1,4 dihydro-4-oxo-piridinyl moiety bearing an essential carbonyl group at position 3. Activity of quinolones is due to the inhibition of prokaryotic type II topoisomerases, namely DNA gyrase and, in few cases, topoisomerase IV.[1] Quinolone derivatives have been shown to inhibit HIV-1 replication in acutely and chronically infected cells.[2–9] Recently, our group developed a new class of 6-substituted quinolones and tested their antibacterial and anti-HIV-1 activities.[10,11] A 6-amino-quinolone, bearing a methyl substituent at the N1 position and a 4-(2-pyridyl)-1-piperazine moiety at the C-7 position (WM5) (Fig. 1A) was found to be the most active compound in inhibiting HIV-1 replication in acutely and chronically infected cells; its mechanism of action involves inhibition of HIV-1 Tat–TAR interactions, resulting in virus life cycle arrest at the Tat-trans-activation level.[12,13] Subsequent structural investigation on a number of analogues of this lead compound permitted us to draw basic structure–activity relationships (SAR);[14] stringent requirements are a small moiety such as methyl or cyclopropyl at position N1, an amino group at C6 and a hydrogen atom at C8. In contrast, position 7 tolerates a variety of substituents, which resulted in the best way to modulate the anti-HIV activity of these quinolone derivatives.

However, among the compounds that exhibited prominent activity compared to the parent drug WM5, quinolone WC12 (28c in[14]) displayed counterbalancing cytotoxicity in CEM cells with dramatic loss of selectivity. This 6-amino-quinolone exhibits a cyclopropyl function at position 1 and a 3-trifluoromethyl-phenyl-piperazine at position 7 (Fig. 1A).

To gain insight into the molecular basis for selectivity displayed by 6-amino-quinolones, we tested the ability of the above compound to interfere with Tat–TAR interaction, recognize specific sequences of nucleic acids, among which TAR RNA structure, and form complexes with magnesium ions. The results were compared to those obtained using the parent potent drug WM5. Interestingly, magnesium binding appears to play a critical role in determining the potency and target selectivity of the test drugs.

2. WC12 inhibits Tat–TAR complex formation

It has been shown recently that the lead of the 6-amino-quinolone series, WM5, is able to inhibit HIV-1

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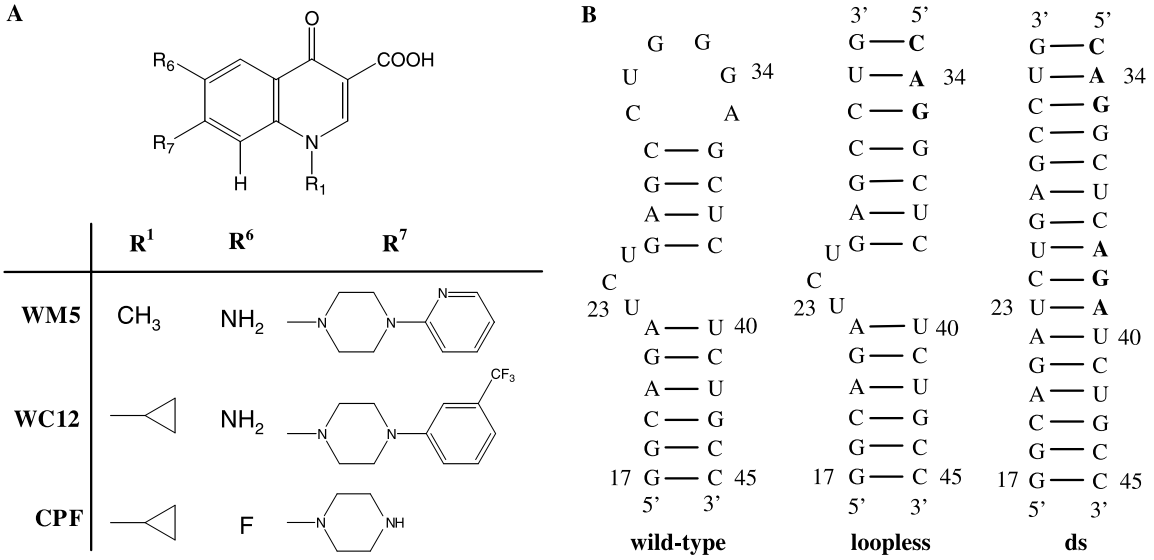

Figure 1. (A) Chemical structures of the test 6-amino-quinolones, WC12 and WM5 and of the control fluoroquinolone, Ciprofloxacin. (B) Secondary structures of wild-type TAR RNA, which spans the minimal sequences that are required for Tat responsiveness in vivo[46] and for in vitro binding of Tat-derived peptides.[15] Loopless TAR is formed by two RNA strands that display complementary bases in the loop portion (the three nucleotides in the loop mutated from the wild-type sequence are shown in bold); ds TAR is constituted by two all-complementary RNA strands (the three inserted nucleotide complementary to the three nucleotide bulge and the three mutated nucleotides in the loop are shown in bold).

replication through specific interaction with the bulge region of TAR RNA (Fig. 1B), which competes for the binding between the viral mRNA and the Tat protein, preventing effective trans-activation.[12] We first investigated whether the test low-selectivity quinolone derivative possessed the same activity. Tat-derived peptides, which contain the basic arginine-rich region of Tat, are able to form in vitro complexes with TAR RNA.[15–19] To achieve specific RNA binding by a Tat fragment, we synthesized a Tat peptide (amino acids 38–72), which contained the RNA-binding domain and 11 amino acids from the core domain of the wild-type sequence of Tat protein. Tat (38–72)–TAR complex formation was assessed by electrophoresis mobility shift assay (EMSA). In these conditions, the affinity of Tat peptide for TAR at 50% of binding is 55 nM.[12] The ability of WC12 to disrupt Tat (38–72)–TAR interaction was measured by titrating a constant amount of Tat–TAR complex (45% of complex on total RNA present in solution) with increasing amounts of the drug. The results are shown in Figure 2. WC12 was able to effectively inhibit Tat–TAR complex formation with an apparent K_i of 3.8 μM, which is in agreement with that previously measured for WM5 (K_i = 3.5 μM).

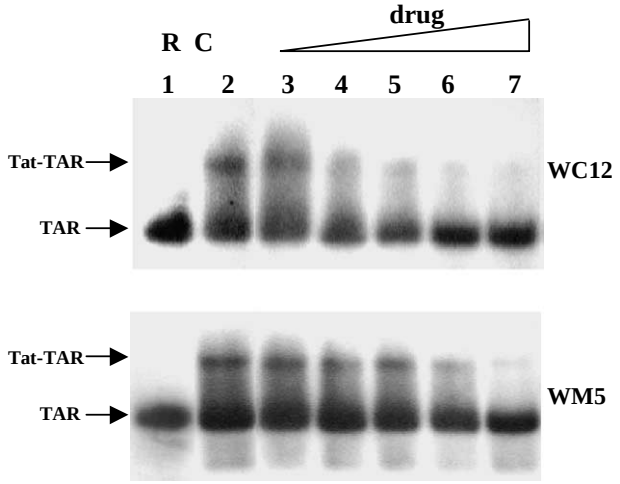

Figure 2. Quinolone inhibition of Tat–TAR interaction. Electrophoretic mobility shift assays show drug inhibition of Tat(38–72)–TAR complex formation. The amounts of TAR RNA and Tat peptide were fixed to 0.2 and 0.1 μM, respectively, in all lanes to get 40% of complex formation on total RNA; the amounts of drugs were 0, 1, 2, 5, 10, and 50 mM in lanes 2–7. Lane C is a control for Tat–TAR complex in the absence of inhibitor; lane R is a control lane for TAR alone. Drug relevance is shown on the right, while band identities (free TAR and Tat–TAR complex) are shown to the left of each gel.

3. WC12 binding to RNA sequences lacks specificity

The above data suggest that the anti-HIV-1 mechanism of WC12 relies on binding to the TAR sequence, as proposed for WM5.[12] To test this hypothesis, we quantitatively measured the RNA-binding properties of the compound. WM5 (Fig. 1A) was included as a control for quinolone with antiviral activity.

The drugs were first assayed for their interaction with wild-type TAR RNA (Fig. 1B). Upon addition of wild-type TAR to WC12 solutions in TNMg buffer, a substantial decrease in the compounds' fluorescence quantum yield was observed, indicating drug–RNA complex formation. In these conditions, the apparent affinity of WC12 to this nucleic acid (EC_{50}), expressed in terms of TAR concentration needed to complex 50% of drug corresponded to 0.19 ± 0.02 (Fig. 3A and Table 1). Hence, WC12 binds to the TAR structure as proficiently as WM5. Subsequently, solutions of the

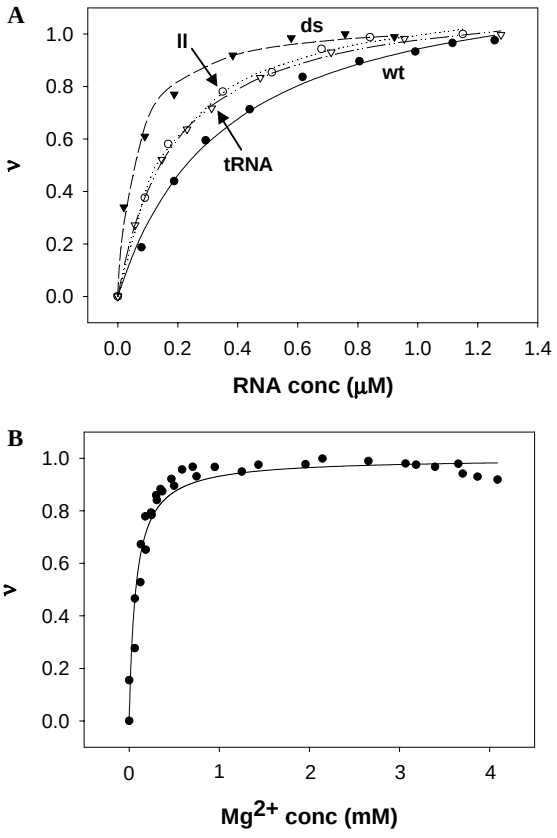

Figure 3. (A) Binding of WC12 to wild-type TAR (wt), loopless TAR (ll), ds-TAR (ds) and tRNA (RNA). Shown is the fraction of bound quinolone (v) versus the RNA concentration. (B) Binding isotherm for WC12–Mg^{2+} complex. The fraction of bound quinolone (v) is plotted against Mg^{2+} concentration. In both cases data were inferred by fluorometric titrations.

Table 1. EC_{50} of 6-amino-quinolone binding to wild-type (wt) and mutant forms of TAR (ll stands for loopless and ds for double-stranded TAR) and tRNA

	WM5	WC12
wt TAR	0.21 ± 0.01	0.19 ± 0.02
ll TAR	0.32 ± 0.02	0.13 ± 0.01
ds TAR	>50	0.050 ± 0.003
tRNA	>50	0.13 ± 0.01

Binding experiments were performed in Tris 10 mM, NaCl 20 mM, $Mg(ClO_4)_2$ 1 mM, pH 7.5, at 25 °C.

test 6-amino-quinolones were titrated with increasing amounts of tRNA. The tRNA was employed as an alternative RNA structure with random sequence to check the selectivity of binding. WC12 showed good affinity for the tRNA also, with EC_{50} of 0.13 ± 0.01 (Fig. 3A and Table 1). The compound was further tested for its binding to mutant structures of TAR, to assess whether the RNA tertiary structure (i.e., bulges, loops, single or double strands) was an important determinant for recognition. The mutant RNAs contained all-annealed RNA segments replacing either the loop (llTAR), or both the loop and bulge regions (dsTAR), maintaining the overall wild-type TAR nucleotide sequence (Fig. 1B). Quantitative evaluation of the fluorescence data gave the EC_{50}s reported in Table 1. WC12 showed the most efficient binding towards the all-double-stranded structure (dsTAR), while affinity towards the other structures was similar (wtTAR, llTAR and tRNA) (Fig. 3A). In contrast, WM5 displayed affinity only for bulge-containing TAR structures.

Quinolones WM5 and WC12 were also investigated in terms of ss- and ds-DNA binding. The corresponding K_i values, calculated using the McGhee and Von Hippel neighbour exclusion model,[20] were of the order of 10^3 M^{-1}, which points to very weak affinity for deoxyribonucleic acids.

4. WC12 efficiently complexes Mg^{2+} ions

The above quinolone–nucleic acid binding experiments were performed at physiological conditions, which include a Mg^{2+} ion concentration of 1 mM. The fundamental role of Mg^{2+} in this type of assays had been previously assessed by a number of studies.[11,21–25] To check the ability of the test drugs to form complexes with the divalent ion, we titrated quinolone solutions with increasing amounts of Mg^{2+}. The binding isotherm referring to WC12 is reported as an example in Figure 3B. Interestingly, and contrary to classic fluoroquinolones,[25] these 6-amino-quinolones were characterized by remarkably high K_{Mg} (Table 2). In particular, WC12 resulted in being the tightest Mg^{2+} binder, followed by WM5 and Ciprofloxacin, used as a control, which exhibited a K_{Mg} at least one order of magnitude lower, in agreement to that reported in the literature at similar experimental conditions.[11]

The structural modification introduced into WC12, while confirming antiviral activity of the 6-amino substituted compound, conferred substantially enhanced cytotoxicity when compared to the parent WM5, decreasing the selectivity index by two orders of magnitude.[14] To understand the molecular basis for modulation of selectivity, we performed a series of measurements comprising binding of the test drug to a number of nucleic acid structures. The results presented support the idea that the anti-HIV-1 activity can be related to interference with Tat–TAR complex formation, as previously indicated for WM5.[12] This possibly is not the only mechanism operating in vivo as the antiviral effect is often multifactorial. Contrary to what occurs with the earlier drug,[12] this action is not the result of a selective recognition of the TAR sequence by WC12, but arises from indiscriminate drug binding to the ribonucleic acid. In particular, the affinity of WC12 for the wild-type TAR sequence is similar to that of WM5, but WC12 binds double-stranded RNA threefold better than wild-type TAR or partly unpaired ribonucleic sequences. These data help explain the poor selectivity and high cytotoxicity reported for this compound. The

Table 2. Binding constants (M^{-1}) for 1:1 quinolone–Mg^{2+} complex in Tris 10 mM, NaCl 20 mM, pH 7.5, at 25 °C

	WM5	WC12	Ciprofloxacin
K (M^{-1})	6650 ± 380	13,600 ± 1120	790 ± 30

test quinolone derivative was also shown to bind both ss- and ds-DNA; however, the binding constants measured were too low to account for cytotoxic effects and, consequently, reduced selectivity indexes.

In the past years it has been extensively demonstrated that Mg^{2+} ions are required for binding of quinolone-based drugs to nucleic acids. In fact, clinically relevant quinolones show negligible affinity for the DNA in the absence of this ion.[11,21–25] The magnesium coordination site is comprised between the carbonyl and carboxyl moieties of the quinolone template and the amount of complexation rests on the protonation equilibria of the different functions present in the drug.[24,26] Our data revealed an excellent ability of the antiviral 6-amino-quinolones, WC12 and WM5, to form complexes with Mg^{2+}. The values obtained in our conditions are remarkably higher than those reported for classical quinolones.[11] The main reason for this is connected to the lower basicity exhibited by the 7 substituent in the antiviral quinolones, as they both exhibit aromatic moieties bound to the piperazine 4′ nitrogen. Hence, the antiviral quinolones are negatively charged at physiological conditions, whereas classical quinolones are globally neutral (zwitterions). This will remarkably favour binding of the former to a positively charged metal ion.

It has been shown that Mg^{2+} can profoundly affect RNA average conformation and plasticity by neutralizing backbone electrostatic repulsions,[27] and thus have important consequences on folding,[28–31] catalysis[32,33] and recognition.[34] Among the many reports on the subject, magnesium has been implied in the stabilization of tertiary structures such as nested double pseudoknots in HDV ribozymes,[35] hairpin loops and pseudoknots,[36–38] and several folded structures, for example, the yeast phenylalanine tRNA[39,40] and the 5S rRNA loop E motif.[41] The importance of the metal ion has also been highlighted for HIV-1 TAR; in particular, while the effect of Mg^{2+} on RNA sequences in the vast majority of examples rests on its ability to unspecifically chelate phosphate oxygens of the ribonucleic acid backbone,[27] in the case of TAR, magnesium binds specifically to the bulge region of this viral RNA[42,43] and it significantly affects both TAR global conformation and dynamics.[44]

It is then conceivable that the ability of WM5 and WC12 in coordinating Mg^{2+} increases their affinity for the bulge region of TAR. Indeed, we have recently shown that WM5 interacts efficiently at this region,[12] and this is confirmed for its analogue in the present work. Therefore, effective chelation to Mg^{2+} located at the bulge represents a plausible requirement for a potent anti-HIV action of 6-amino-quinolone.

As far as selectivity is concerned, drug action must be preferential to avoid undesired side toxicity. Hence, drug affinity for Mg^{2+} should not be exceedingly high to display an optimum selectivity index. In fact, when the K_{Mg} increases with reference to WM5, as in the case of WC12, drug binding becomes less specific. W12 (K_{Mg} higher than WM5) maintains a high affinity for the wild-type form of TAR, but it also shows a relatively high affinity for mutant TARs; in fact, the tightest binding is displayed against a double-helical RNA that is where backbone negative charges are more concentrated.

Interestingly, Ciprofloxacin, which is inactive as an anti-HIV compound, shows no detectable tendency to bind TAR or other RNA targets.

In conclusion, structural modifications at position 7 of the 6-amino-quinolone template have a dramatic effect on the potency and selectivity of these drugs. In this connection, we propose that the basicity of the substituent at position 7 modulates binding to magnesium, producing changes in RNA binding and, consequently, in selectivity indexes observed with the various drugs. Low K_{Mg} values, as in the case of classical quinolones (e.g., ciprofloxacin), induce poor interaction with all RNA sites, including the bulge of TAR.[12] Hence these drugs do not exhibit anti-HIV properties. Increasing K_{Mg}, as it occurs for WM5, allows binding to specific sites in the RNA structures (e.g., TAR), but prevents generalized complexation, hence drug-induced damage, to other non-viral sites. This accounts for the remarkably high selectivity index exhibited by this compound. A further increase in K_{Mg} (WC12) not only enhances TAR binding, but also promotes drug interactions with several other viral and cellular RNA structures, thus producing higher anti-HIV responses together with simultaneous toxic effects to the host cell. However, this explanation, which suggests a relationship between Mg complexation, RNA binding and antiviral/cytotoxic activity, requires testing of a larger number of compounds, to be safely established.

A final consideration is that highly effective binding of WC12 to duplex structures of RNA could be exploited to selectively direct this drug towards targets having ds-RNA as a key intermediate in therapeutically relevant processes.

Quinolone stock solutions (1 mg/mL) were made in DMSO and diluted to the working concentration in the desired buffer. All TAR RNAs were purchased from Dharmacon (Lafayette, USA). TAR sequence were: 5′-GGCAGAUCUGAGCCUGGGAGCUCUCUGCC-3′ (wild-type); 5′-GGCAGAUCUGAGCCUG-3′ (loopless-1) and 5′-CAGGCUCUCUGCC-3′ (loopless-2); 5′-GGCAGAUCUGAGCCUG-3′ (ds-1) and 5′-CAGGCUCAGAUCUGCC-3′ (ds-2). TAR–Tat peptide binding reactions (20 μL) containing 4 pmol of 5′ end-labeled RNA and increasing amounts of Tat (38–72) (0.01–50 μM final concentration),[12] in TNMg buffer (10 mM Tris–HCl, pH 7.4, 20 mM NaCl and 1 mM $MgCl_2$) were incubated at 25 °C for 30 min. For inhibition assays the amount of TAR RNA and Tat peptide was fixed at 0.2 and 0.1 μM, respectively, to get a 50% complex formation; the amount of each drug was gradually increased up to 50 μM. Complexes were separated from unbound TAR RNA by electrophoresis in non-denaturing 10% polyacrylamide gels.

For RNA binding, fluorescence spectra were recorded at 25 °C in Tris–HCl 10 mM, pH 7.0, NaCl 20 mM and

$Mg(ClO_4)_2$ 1 mM. The fraction of bound drug (v) was obtained as reported previously.[45] The DNA binding constants were calculated using the McGhee and Von Hippel neighbour exclusion model.[20] For Mg^{2+} binding, quinolone fluorescence intensity was measured in Tris 10 mM, NaCl 20 mM, pH 7.5, 25 °C, as described previously.[11,25]

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