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Synthesis and biological evaluation of 2-phenylquinolones targeted at Tat/TAR recognition

Giuseppe Manfroni [a], Barbara Gatto [b,*], Oriana Tabarrini [a], Stefano Sabatini [a], Violetta Cecchetti [a], Giulia Giaretta [b], Cristina Parolin [c], Claudia Del Vecchio [d], Arianna Calistri [d] Manlio Palumbo [b], Arnaldo Fravolini [a]

[a] Dipartimento di Chimica e Tecnologia del Farmaco, Università di Perugia, Via del Liceo 1, 06123 Perugia, Italy
[b] Dipartimento di Scienze Farmaceutiche, Università di Padova, Via Marzolo 5, 35131 Padova, Italy
[c] Dipartimento di Biologia, Università di Padova, Via Ugo Bassi 58/B, 35131 Padova, Italy
[d] Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche, Università di Padova, Via Gabelli 63, 35121 Padova, Italy

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ABSTRACT

Tat (transactivator of transcription) is a small HIV protein rich in arginines that interacts with a viral RNA structure called TAR (trans-activation responsive region). Tat–TAR interaction is essential for viral gene expression, replication and pathogenesis. Small molecules able to interfere with TAR and to compete for Tat binding possess antiviral activity due to inhibition of viral transcription and expression, thus impairing formation of infectious virions. We report here, the synthesis and biological evaluation of a new series of quinolone derivatives, namely 2-phenylquinolones, designed with the aim of interfering with the protein/RNA complex. These new derivatives are able to efficiently interfere with Tat/TAR complex in vitro depending on precise structural requirements as demonstrated by fluorescence quenching assay analysis.

The trans-activation response element (TAR) RNA from human immunodeficiency virus type 1 (HIV-1), is a 59-base stem–loop structure located at the 5′ end of all viral transcripts[1] that plays a crucial role in the HIV life cycle. TAR has gained increased importance as a therapeutic target for small-molecule drug discovery since it became clear that its interaction with the cognate transcriptional activator protein, Tat,[2] drastically increases the processivity of RNA polymerase II.[3] Tat/TAR interaction in fact results in an exponential increase in the production of viral transcripts and expression of all proteins necessary to complete the HIV life cycle.[4,5] By blocking this interaction the viral transcription and expression is inhibited, thus providing a potential route for AIDS chemotherapy.[6] The discovery of transcription (transactivation) inhibitors is an innovative line of investigation in medicinal chemistry. These inhibitors are thought to control HIV-replication not only in acutely infected cells but also in chronically infected ones leading to complete eradication of the viral infection.[7] In addition, due to the high conservation of both Tat and TAR sequences, the identification of an inhibitor of Tat/TAR complex would guarantee binding specificity and help to reduce the toxicity associated with the currently used drugs.

The minimal TAR RNA sequence sufficient for Tat responsiveness in vivo is located from nucleotides +19 to +42;[8] its secondary structure is characterized by a six-nucleotide loop and a three-nucleotide pyrimidine bulge which separates two helical stem regions.[9] The bulge is essential for high affinity and specific binding of the Tat protein. Among the Tat/TAR complex inhibitors, a limited number of small synthetic molecules bind the TAR/RNA directly to the three-base bulge or to the bulge along with the TAR stem–loop region.[10] All these compounds, whether the result of virtual screening or rational design, fit a common model which entails (i) an aromatic or heteroaromatic portion, with the potential for stacking interactions in the bulge, (ii) one or two cationic residues providing electrostatic interactions with the phosphate backbone of the RNA, and (iii) a flexible side chain of optimal length that links these two moieties.[10]

We recently identified potent anti-HIV 6-desfluoroquinolones which owe their antiviral action to the inhibition of Tat-mediated transcription.[11–15] In particular, the lead compound of the series, WM5, 6-amino-1-methyl-4-oxo-7-[4-(2-pyridyl)-1-piperazinyl]-1,4-dihydroquinolin-3-carboxylic acid (**1**),[11] was able to disrupt the Tat/TAR complex with 50% inhibitory concentration (IC_{50}) in the low micromolar range.[12] The extensive structure–activity relationship (SAR) studies conducted on this series showed that higher antiviral potency was often accomplished at the price of increased cytotoxicity. Since loss of selectivity was linked to increased

* Corresponding author. Tel.: +39 049 8275717; fax: +39 049 8275366.
E-mail address: barbara.gatto@unipd.it (B. Gatto).

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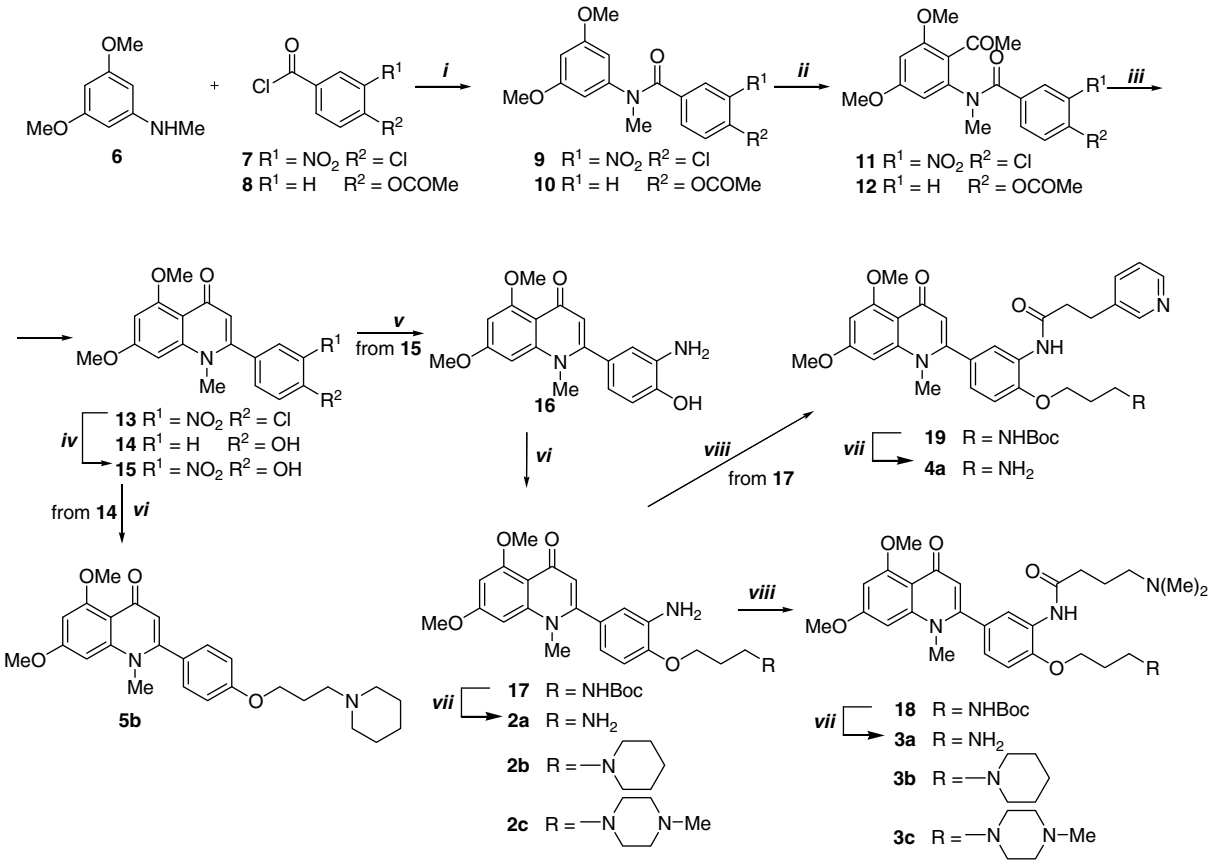
magnesium binding by the 4-keto-3-carboxylic group of quinolones, resulting in non-specific binding to nucleic acids,[14] our search for new small molecules as specific inhibitors of the Tat/TAR complex is aimed at compounds devoid of this moiety, essential for chelation of magnesium ion in the classical pharmacophoric model of quinolones.[16] A new series of quinolones lacking the C-3 carboxylic function were therefore designed following the above mentioned structural requirements for TAR recognition[10] and were significantly shaped by the findings reported for the bi-aryl heterocyclic molecules ("rbts") emerged from HTS screening for Tat–TAR competitors.[17] Hence, we synthesized and tested 2-phenylquinolone derivatives **2a–c**, **3a–c**, **4a**, and **5b** (Scheme 1), where the quinolone ring would serve as the stacking moiety between the base pairs of the nucleic acid,[18] and as scaffold for the phenyl ring bearing cationic substituents. In particular, compounds **2** are characterized by a mono-*O*-propyl protonable side chain with compound **5b** lacking also the vicinal NH_2 group, while compounds **3** and **4** bear a second protonable side chain. Compound **16** lacking both side chains was also included in the biological evaluation to verify the need for cationic substituents.

Our goal is to (i) verify whether modifications at the 3,4-chelating moiety of quinolones allow activity in the TAR system and (ii) explore the requirements for phenyl ring substituents for efficient recognition of the Tat/TAR complex.

The synthesis of the target compounds **2a–c**, **3a–c**, and **4a** initially entailed the preparation of the suitable functionalized 2-phenylquinolone **16** (Scheme 1). Condensation of *N*-methyl-3,5-dimethoxyaniline, **6**[19] with 4-chloro-3-nitrobenzoyl chloride **7**, gave the amide intermediate **9**, which by Friedel–Crafts acylation using $SnCl_4$ as a catalyst, afforded the desired regioisomer **11** in 64% yield. The successive cyclization with *t*-BuOK gave the 5,7-dimethoxy-1-methyl-2-phenylquinolone **13** which by treatment with KOH at 90 °C, followed by the nitro group reduction, afforded the 2-phenylquinolone scaffold **16**. For the preparation of the aminoderivative **2a**, compound **16** was reacted with *t*-butyl-3-bromopropyl carbammate,[20] to give intermediate **17** which was then deprotected using trifluoroacetic acid (TFA). The *O*-propyl derivatives **2b** and **2c** were prepared directly by reacting **16** with 1-(3-chloropropyl)piperidine (**b**)[21] and 1-(3-chloropropyl)-4-methylpiperazine (**c**)[22] respectively, in the presence of Cs_2CO_3. Di-substituted derivatives **3b** and **3c** were prepared by reacting **2b** and **2c**, respectively, with 4-(dimethylamino)butyric acid (DMABA) in the presence of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDAC) and 4-dimethylaminopyridine (DMAP). The same reaction was carried out starting from intermediate **17** to give amide **18**, which was then deprotected to di-substituted derivative **3a**. Starting from intermediate **17**, derivative **4a** was also prepared by reaction with 3-(3-pyridyl)propionic acid followed by deprotection. Following an analogous synthetic route, desamino derivative **5b** was obtained by reacting aniline **6** with 4-(chlorocarbonyl)phenyl acetate **8**, through intermediates **10**, **12**, and **14** (see Supplementary material for a detailed description of synthetic procedures).

The ability of all the designed 2-phenylquinolones **2–5** and **16** to inhibit the Tat/TAR complex formation was tested by Fluorescence Quenching Assay (FQA) employing the basic peptide (residues 48–57: GRKKRRQRRR) spanning the minimal region necessary for TAR binding.[23,24] The protocol envisages the use of TAR labelled with a quencher moiety (Q) and of Tat labelled with a fluorescent dye (D). The fluorophore emits efficiently when the



Scheme 1. Reagents and conditions: (i) Et_3N, THF, 0 °C; (ii) MeCOCl, $SnCl_4$, CH_2Cl_2, 0 °C; (iii) *t*-BuOK, *t*-BuOH, 30 °C; (iv) KOH/H_2O, DMSO, 90 °C; (v) H_2, Raney Ni, DMF/2-methoxyethanol; (vi) $R(CH_2)_3X$, Cs_2CO_3, DMF, 50/70 °C; (vii) TFA, CH_2Cl_2; (viii) DMABA or 3-pyridin-3-yl propanoic acid, EDAC, DMAP, DMF.

peptide is free in solution, but its emission dramatically decreases when in close proximity to the quencher dabcyl molecule, that is, when the QD complex is formed. Interference on Tat/TAR complex formation in the presence of fixed concentration of quinolones is clearly seen by the increase in fluorescence signal respect to what observed in the peptide/nucleic acid titration. These measures allow us to determine the inhibition constants for each compound, reported in Table 1.[25]

Compounds **2b, 2c**, and **5b** show inhibition constants comparable or lower of the positive control (**1**) while all other derivatives are less or totally inactive. Removal of all cationic side chains is totally detrimental to activity, as demonstrated by the absence of inhibition exhibited by **16**. Notably, **2b** and **2c** have only one basic side chain, while the corresponding phenylquinolones **3b** and **3c** bearing two cationic side chains are less active or inactive in these experimental conditions. The lower or null activity of the two-side-chain compounds is also evidenced by the K_i values of **3a** and **4a**. The low activity shown by **2a** points to the key importance of a nitrogen containing heterocycle as protonable head in the mono-side-chain derivatives. Having established that cationic substituents in meta of the phenyl ring are detrimental to Tat/TAR inhibition activity, we tested whether removal of any substituent at this position affected activity. Very interestingly, compound **5b**, the desamino analog of the mono-side-chain **2b**, is the most active derivative of all 2-phenylquinolones, indicating that this position could be free. While steric and electronic contribution may be taken in account, it is tempting to speculate that in **2b** an intramolecular H-bond between the amino and the oxygen of the ether bridge forces the orientation of the cationic side chain in a less favored position for nucleic acid/peptide recognition.

The antiviral activity of the compounds able to interfere with Tat/TAR interaction in FQA assays was evaluated using Jurkat cells infected with laboratory-adapted HIV-1 strains. The virological parameters considered were the reverse transcriptase (RT) activity using specific template/primer. At 11, 18, and 21 days post-infections of Jurkat cells, the RT titer of the drug treated cells (1–100 μM) was only marginally lower compared to the untreated control at all concentrations tested (see Supplementary data). Furthermore, the cytotoxicity was evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: none of the compounds showed any cytotoxicity up to 100 μM in CEM as well as in Jurkat cell lines, with the exception of **5b**, showing a residual cytotoxicity in Jurkat cells after 72 h of incubation (EC_{50} = 94.7 ± 14.4 μM). We therefore wondered if the lack of cytotoxicity and antiviral activity in infected cells exhibited by the 2-phenylquinolones compounds might be related to an unfavorable pharmacokinetic profile. These new derivatives in fact are highly hydrophilic and dissolve easily in aqueous buffers, as expected from highly charged compounds. We hence measured the uptake of selected 2-phenylquinolones by cultured cell lines to verify cell penetration for compounds **2b**, **2c**, and **5b** exhibiting in vitro activity, using **3a** and **16** as negative and **1** as positive controls. The amount of compound found in the cell lysate after extensive washing (% uptake) was measured at different times of incubation and did not improve after 3 h, neither using different cells densities. At the best experimental conditions, the percentages of quinolones found in the cell lysate are shown in Table 1.[26]

Table 1
Biological evaluation of 2-phenylquinolone derivatives

Compound	K_i[a] (μM)	% Uptake[b] (Jurkat cells)	% Uptake[b] (CEM cells)
2a	3.85 ± 0.86	ND[d]	ND[d]
2b	1.95 ± 0.50	0.94	1.80
2c	1.58 ± 0.29	0.82	1.10
3a	3.03 ± 0.51	0.67	1.20
3b	2.60 ± 0.43	ND[d]	ND[d]
3c	NA[c]	ND[d]	ND[d]
4a	NA[c]	ND[d]	ND[d]
5b	1.02 ± 0.12	3.27	ND[d]
16	NA[c]	25	ND[d]
1	2.18 ± 0.34	23.2	7.50

[a] K_i from FQA experiments performed with 10 aa FAM–Tat peptide in Tris 10 mM, pH 7.5, $Mg(ClO_4)_2$ 1 mM, NaCl 20 mM (TNMg), 0.01% Triton X-100.
[b] Uptake experiments performed after 3 h of incubation at 37 °C with the indicated cell lines.
[c] NA, not active.
[d] ND, not determined.

It is clearly evident by these data how our positive control **1** shows a significant ability to be taken up by the cells, in particular by the Jurkat cells employed for viral infections, its uptake percentage improving significantly, up to 40% by preincubating Jurkat cells with the cationic detergent Lipofectamine (not shown). The amount of all 2-phenylquinolones found in uninfected cells is on the opposite very low, especially in Jurkat cells, at all conditions. Compound **16**, bearing no charged substituents at the phenyl ring and no in vitro activity, is on the opposite the one with the highest cell permeation profile. This data point out how the requirements for activity in Tat/TAR system are also those unfavoring cellular permeation. The remarkable uptake of **1** clearly allows activity in infected cells, while the charged nature of 2-phenylquinolones derivatives is detrimental to cell penetration. The unfavorable pharmacokinetic parameters therefore likely account for the lack of their activity and toxicity in cell lines, despite the good profile of activity in Tat/TAR inhibition assays.

In conclusion, we report here the design, synthesis, and biological activity of novel quinolones characterized by the lack of the usual C-3 carboxyl moiety and by the presence at the C-2 position of a phenyl group functionalized with different protonable side chains. These new small molecules are able to efficiently interfere with Tat/TAR complex in vitro depending on precise structural features. A clear important conclusion of our structure–activity analysis is the requirement of only one basic side chain at the *para* position of the phenyl ring for competition of Tat/TAR binding, differently from the series of bi-aryl-heterocycles[17] chosen as model for the design of our phenylquinolones. In that case it was clearly demonstrated how TAR binding is achieved in the presence of two essential cationic substituents at the phenyl ring.[17]

Our finding has also favorable implications, since it impacts well on pharmacokinetic considerations. Although **5b**, **2b**, and **2c** in vitro data are similar or better than those found for a classical quinolone as **1**, it is evident that the cell parameters are profoundly different, reflecting the ionic nature of 2-phenylquinolone derivatives, devised from the pharmacophoric model of RNA-binding compounds.[10] However, the 2-phenylquinolones bearing a single basic side chain have reduced molecular mass than the inactive analogs, and are also those exhibiting reduced overall charge. The analysis of in vitro data coupled with uptake studies therefore gives us a clear indication on the requirements for antiviral activity by the new 2-phenylquinolones. Namely, we will proceed exploring the activity of novel compounds exhibiting only one side chain but whose lipophilicity may be improved by variations at the quinolone nucleus as well as by modifications of the substituents at the 2-phenyl ring.

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Supplementary data

Experimental details corresponding to the synthesis and analytical data of compounds **2a–c, 3a–c**, **4a**, **5b** and the protocol for antiviral evaluation in HIV-infected cells can be found, in the online version, in the Supplementary material. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.034.

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25. Titrations of labelled Tat/TAR in the presence of competitors were made in triplicates in a 96-well plate reader (Victor III, Perkin-Elmer); the fluorescein-labeled peptide (FAM–Tat) was excited at 490 nm and the emission was recorded at 535 nm. The plates were assembled with 190 μl of a solution containing 10 nM fluorescein-peptide in Tris 10 mM, pH 7.5, $Mg(ClO4)_2$ 1 mM, NaCl 20Mm, 0.01% Triton X-100 (TNMg), in presence of fixed amounts of each compound (10 μM). Fluorescence intensities in the presence of increasing concentrations of dabcyl-TAR were measured and curves were fitted to obtain K_i. A preliminary determination of the spectral parameters of all quinolones allowed us to exclude optical interferences with fluorescein-peptide signal at the conditions of the assay.
26. Jurkat or CEM cells (2.5×10^6/well) were incubated in HBSS (GIBCO) with the quinolones at the concentration of 100 μM. After three hours of incubation at 37 °C, cells were separated from the extracellular solution by centrifugation through a water-impermeable silicone–oil barrier (density, 1029 g/cm^3) in a microcentrifuge tube. The cell pellet was lysed overnight with 1 mL of 0.1 M glycine–HCl buffer (pH 3.0) and then the samples were centrifuged for 5 min at 5600*g*. The amount of compound in the lysis supernatant was determined by fluorescence directly in glycine buffer at pH 3, conditions at which it is possible to experimentally determine a titration curve for each quinolone. Controls without quinolones were always used to determine the background fluorescence.