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New Chloroquine Analogues as Antiviral Agents

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SUMMARY. Rhinoviruses and Enteroviruses (polio, coxsackie, echovirus) are the two types of picornaviruses responsible for the high number of human viral infections as the common cold. Structure Activity Relationship data about antiviral agents indicate the nitrogened heterocycles are present in several active compounds. Considering the antiviral activity of chloroquine, used as standard drug for the Coxsackievirus B-3, two new analogues were synthesized and evaluated as antiviral agents: N^4-(3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine)-N^1,N^1-diethyl-1,4-pentanediamine [1] and N^4-(quinaldine)-N^1,N^1-diethyl-1,4-pentanediamine [2]. The compound [1] showed *in vitro* antiviral activity against Coxsackievirus.

RESUMEN. "Nuevos Análogos de la Cloroquina como Agentes Antivirales". Rinovirus y Enterovirus (polio, coxsackie, echovirus) son los dos tipos de picornavirus responsables del alto número de infecciones virales en humanos como el resfrío (o la gripe). Datos de Relación Estructura Actividad sobre antivirales indica que los heterociclos nitrogenados están presentes en la mayoría de los compuestos activos. Considerando la actividad antiviral de cloroquina, usada como droga estándar para el Coxsackievirus B-3, se sintetizaron y evaluaron dos análogos como agentes antivirales: N^4-(3-metil-1-fenil-1H-pyrazolo[3,4-*b*]piridina)-N^1,N^1-dietil-1,4-pentanediamina **[1]** y N^4-(quinaldina)-N^1,N^1-dietil-1,4-pentanediamina **[2]**. El compuesto **[1]** mostró actividad *in vitro* contra Coxsackievirus.

INTRODUCTION

Viruses are microscopic parasitic organisms which infect all living cells. Viruses cause minor as well as major infections in the host. Acute viral respiratory infections are the most common cause of the morbity around the world. Adenovirus, orthomyxovirus, paramyxovirus and picornavirus family (rhinoviruses and enteroviruses) are responsible for causing respiratory diseases. Some of the common diseases associated with such viruses are acute viral rhinitis, pharyngitis, laryngotracheobronchitis, pneumonitis, influenza and parainfluenza [1,2].

The picornaviruses share structural features in their capsids. Their classification is based on physical properties (particle density and pH-sensitivity), serological relatedness and nucleotide sequence. There are 5 genera: aphthovirus, cardiovirus, enterovirus, hepatovirus and rhinovirus. The enteroviruses genera are the most frequently detected etiologic organisms, which was traditionally divides into tree groups (polio, cosackie and echoviruses). The virus causing hepatitis A (hepatovirus) has similar and biological characteristics to the enteroviruses. They are non-enveloped RNA viruses that are stable in acidic pH [1,3,4].

The highest incidence of enteroviruses, especially the coxsackieviruses, detected in infection human is the common cold. Considering the worldwide diffusion of respiratory diseases, these syndromes have relevant socioeconomic importance. Many efforts have been directed toward the identification of agents useful in their prophylaxis and therapy [1]. Nevertheless, at the present time, no therapeutic agents is licensed

KEY WORDS: Antiviral agents, Chloroquine, Coxsackievirus, Heterocycles.
PALABRAS CLAVE: Antivirales, Cloroquina, Coxsackievirus, Heterociclos.

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for the treatment of infection caused by any of the picornaviruses.

Antiviral agents not only interfere with viral replication processes but also affect the host's normal cellular metabolic process. Most drugs are unsuccessful due either their toxicity to the host cell or because they are not effective enough to treat a variety of viral infections at therapeutic dose levels.

One class of antiviral agents which has demonstrated activity against picornaviruses are the capsid-binding compounds [5]. The [(oxazolylphenoxy)alkyl] isoxazoles, disoxaril and WIN 54954, have demonstrated a wide broad-spectrum activity *in vitro* against several picornaviruses and are effective *in vivo* to mice infected with polio, echo and coxsackie viruses [5-10].

Antipicornaviral molecules are hydrophobic and exhibit little solubility in aqueous-based biological systems [7,10]. Structure activity relationship data of antiviral agents showed that *N*-heteroaromatic rings represent an important substructure in a variety of antiviral agents [2,11,12].

The chloroquine (**[3]**, Fig. 1) a quinoline derivative generally used in the therapy of malaria, has been shown antiviral properties against some viral infections. Of the particular interest for human pathology is the inhibition of hepatitis A virus [12-14].

In view of the chemical instability associated with the oxazoline ring [6,7] and considering the antiviral activity of chloroquine, the last one was used as standard drug for the Coxsackievirus B-3. We report the synthesis and antiviral properties of the 1-H-pyrazolo[3,4-*b*]pyridine (1) and quinaldine (2) analogs, which are structurely related to chloroquine (Fig. 1).

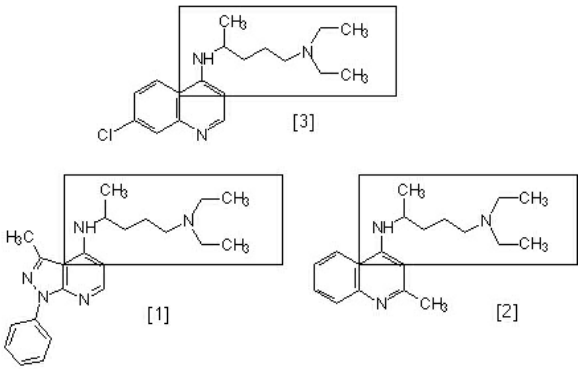


Figure 1. Structural relationship between chloroquine [3] and the new heterocyclic analogs [1] and [2] (pyrazolopyridine analog and quinaldine analog, respectively).

MATERIAL AND METHODS

Chemistry

Electron impact mass spectra were determined using a GC/VG Micromass 12 to 70 eV and a Hewlett Packard gas chromatograph (HP 5890) with mass specter detector [MSD (HP 59708)], column DB-1, 15 m, ID 0.25mm, at initial temperature of 170(C (1min), increasing 30 °C/min and final temperature 250 °C, injector temperature 250 °C. NMR spectra were recorded on a Varian VXR-300 spectrometer. The identification of the compounds on ^{13}CNMR were aided by APT spectra. IR spectra were recorded on a Perkin-Elmer 281b IR spectrophotometer by using potassium bromide plates. Melting points were determined on a Thomas Hoover PC03296 apparatus and are uncorrected. Organic extracts were dried over anhydrous sodium sulfate and evaporated at reduced pressure. Chromatography was performed on Merck-Darmstadt silica gel 230-400 mesh. The progress of all reactions was monitored by t.l.c. performed on Kilsegel 60F 0.2 mm (Merck-Darmstadt). The developed chromatograms were viewed under ultraviolet light (254-365 mm).

Biology

The screening was based on inhibition of cytopathic effects of the viruses in the Vero cell culture system, infected with Coxsakievirus B-3 (RNA, non enveloped, ATCC #VR30). Vero (African green monkey kidney) cell was grown to near confluence in RPMI 1640 with 10% calf serum, then inoculated with 5 times the viral dose necessary to destroy 50% of the cell monolayer. Following 1 h for viral absorption, medium is added, containing test samples at nominal concentrations of 500, 150 and 50 µg/mL. Some wells receive vehicle (DMSO) or positive antiviral agent (chloroquine). Uninfected wells were run in parallel, and serve as citotoxicity controls [15]. Incubation were carried out for 48 h, following which the cell viability in the wells is estimated by staining with neutral red is added, and incubation is continued for 1 h. Wells were washed with warm phosphate-buffered saline, then the incorporated dye is released from the cells by addition of 200 µL isopropanol/0, 4N HCl. Plates are read at 490 nm on a Biotek 312 EL plate reader.

Data were analyzed utilizing macros in Microsoft Excel, with comparisons to standard antiviral drugs. Samples which show at least 30% inhibition of the viral cytophatic effects were retested for confirmation.

Experimental procedures

Ethyl-5-[3-methyl-4-(N^1,N^1-diethyl-1,4-pentanediamine)-1-phenyl-1-H-pyrazolo[3,4-b] pyridinyl]carboxylate [5]

To a suspension of the ethyl-5-[4-chloro-3-methyl-1-phenyl-1-*H*-pyrazolo[3,4-*b*] pyridinyl] carboxylate **[4]** (0,5 g; 1,6 mmol) in toluene (10 mL) was added N^1,N^1-diethyl-1,4-pentanodiamine (0,7 mL; 3,2 mmol), and the mixture was refluxed under nitrogen atmosphere for 2 h. The reaction mixture was concentrated under reduced pressure to furnished an yellow oil. It was purified by aluminium column chromatography (hexane) to give **[5]** (41%). ^{1}HNMR, 300 MHz ($CDCl_3$) δ: 0.94 (t, J=7.2 Hz, 6H, CH_3CH_2N); 1.36 (q, J=7.2 Hz, CH_2); 1.47 (m, unresolved, CH_3CH); 1.58-1.65 (m, unresolved, CH_3CH & CH_2N); 2.35 (t, J=7,3 Hz, 2H, OCH_2CH_3); 2,42 (q, J=7,2 Hz, 4H, $N(CH_2CH_3)_2$); 2.73 (s, 3H, H-3); 4.32 (q, J=7.2Hz, 2H, CH_3CH_2O); 7.28 (m, unresolved, 1H, H-4'); 7.47 (ddd, 2H, J =8.1, 7.7, 1.9Hz, H-3'); 8.06 (dd, 2H, J=7.5, 1.2Hz, H-2'); 8.91 (s, 1H, H-6); 9.03 (d, J=9.8Hz, NH). ^{13}CNMR, 75MHz ($CDCl_3$) δ: 11.48, 14.19, 18.15 e 21.91 (CH_3); 23.56, 36.63, 46.72, 52.62 e 60.45 (CH_2); 53.14 (CH alkyl); 102.31 (C-3a); 105.10 (C-5); 122.27 (C-2'); 126.17 (C-4'); 128.86 (C-3'); 142.18 (C-1'e C-3); 153.01 (C-7a); 154.11 (C-4); 154.75 (C-6); 169.05 (C=0). APT. CH_3: δ 53.14, 21.91, 18.15, 14.19, 11.48C; CH_2: δ 60.45, 52.62, 46.72, 36.63, 23.56; CH: δ 153.01, 128.86, 126.17, 122.27; C: (169.05, 154.75, 154.11, 142.18, 138.86, 105.10, 102.31 MS: m/z (rel. int.) 437 (M+,14%), 408 (4), 363 (4), 297 (4), 277 (9), 112 (7), 99 (11), 86 (100), 83 (30), 77 (3), 73 (5), 58 (10).

3-Methyl-4-(N^1,N^1-diethyl-1,4-pentanediamine)-1-phenyl-1-H-pyrazolo[3,4-b]pyridine-5-carboxylic acid [6]

A solution of 5 (0.3 g, 0.66 mmol), 20% NaOH (5 mL) and ethanol (5 mL) was heated to reflux for 1h. After cooled, the reaction mixture was neutralized with 10% HCl, then it was taken up in ethyl acetate. To the water layer was added small quantity of sodium chloride to precipitate white crystals. The product was filtered off, washed with cold water, and dried.

IR (KBr) γ-cm^{-1}: 3500-3300 (NH_2^+); 1580 (CO_2^-). ^{1}HNMR, 300 MHz ($CD_3OD/CDCl_3$) δ: 1.23 (t, J=7.3Hz, CH_3CH_2N); 1.31 (d, J=6.5Hz, $CHCH_3$); 1.68 (m, unresolved, 4H, 2 CH_2); 2.69 (s, 3H , H-3); 3,06 (m, unresolved, 6H, CH_2-N); 3.31 (m, unresolved, 1H, $CHCH_3$); 7.30 (dd, 1H, J=7.4, H-4'); 7.47 (ddd, 2H, J=8.2, 8.2, 1.6Hz, H-3'); 7.88 (dd, 2H, J=7.4, 1.2Hz, H-2'); 8.9 (s, 1H , H-6) MS: m/z (rel. int.) 409 (M+, 10%), 335 (5), 277 (7), 269 (5), 167 (4), 140 (4), 99 (8), 86 (100), 77 (4), 73 (4), 58 (7).

N^4 -(3-methyl-4-(N1,N1-diethyl-1,4-pentanediamine)-1-phenyl-1-H-pyrazolo[3,4-b] pyridine hydrochloride [1]

A solution of **[6]** (0.14 g, 0.34 mmol) and phosphoric acid (5 mL) was heated to reflux for 12 h. After cooled, the reaction mixture was taken up in ice-cold water and neutralized with 10%NaOH. The product was extracted with ethyl acetate, and a few drops of conc. HCl were added. The solvent was evaporated and the residue was recrystallized from ethanol/ethyl acetate to furnished 1 in 66% yield.

^{1}HNMR, 300 MHz ($CD_3OD/CDCl_3$): δ 1.14 (t, J=7.0Hz, CH_3CH_2N); 1.30 (m, unresolved, CH_3-CH); 1.38 (m, unresolved, CH_2); 1.9 (m, unresolved, 3H, CH_2 & CH_3-CH); 2.81 (s, 3H, H-3); 3.60 (q, J=7.0Hz, 2H, NCH_2); 6.65 (1H, H-7); 7.30 (m, unresolved, H-4'); 7.46 (dd, J=7.5 e 7.9Hz, H-3'); 7.58 (d, J=7.8Hz, H-2'); 8.05 (1H, H-6); 10.96 (broad peak, NH_2). ^{13}CNMR, 75 MHz ($CD_3OD/CDCl_3$): δ 8.53, 8.66 (CH_3-CH_2), 15.51 (C-8), 20.29 (CH_3-CH(NH)); 21.10, 32.85 (CH_2); 46.72, 51.86, 57.86 (CH_2-N); 50.24 (CH(NH) CH_3); 105.65 (C-5); 98.56 (C-3a); 123.02 (C-2'); 128.44 (C-4'); 129.79 (C-3'); 136.00 (C-3); 139.89 (C-1'); 140.51 (C-7a); 144.77 (C-6); 153.92 (C-4) APT CH_3: δ 20.29, 18.23, 15.51, 8.66;); CH_2: δ 57.86, 51.86, 46.90, 32.85, 21.10; CH: δ 139.89, 129.79, 128.44, 123.02, 98.56, 50.24; C: δ 153.92, 144.77, 140.51, 136.00, 105.65 MS: m/z (rel. int.) 365 (M+,14%), 336 (9), 291 (6), 251 (6), 224 (6), 86 (100), 77 (4), 73 (5), 58 (7).

4-(N^1,N^1-diethyl-1,4-pentanediamine)-quinaldine succinate [2]

A solution of 4-chloro-quinaldine **[7]** (1 g, 5.6 mmol) and N^1,N^1-diethyl-1,4-pentanediamine (2,5 mL; 12,9 mmol) was heated to reflux under nitrogen atmosphere for 5 hours. The reaction mixture was taken up in water, extracted with ethyl acetate, dried and poured in florisil column twice. To the solution was added succinic acid and concentrated under reduced pressure.

^{1}HNMR, 300 MHz ($CDCl_3$): δ 0,98 (t, J=7,2Hz, 6H, CH_3CH_2N); 1,28 (d, J= Hz, 3H, CH_3-CH); 1,59 (m, unresolved, 4H, 2 CH_2); 2,48 (m, unresolved, 6H, NCH_2); 2,58 (s, 3H, CH_3-Ar); 3,69 (m, unresolved, 1H, CH_3-CH); 5,03 (1N, NH); 6,31(s, 1H, H-3); 7,32 (ddd, J=7,6Hz, 7,6Hz, 1,3Hz, 1H, H-6); 7,56 (ddd, J=7,6Hz, 7,7Hz,

1,4Hz, 1H, H-7); 7,66 (dd, J=7,8Hz, 1,1Hz, 1H, H-5); 7,87 (dd, J=8,0Hz, 0,9Hz, 1H, H-8). ^{13}CNMR, 75MHz ($CDCl_3$): δ 11,40 & 20,24 ($_2(CH_3CH_2)N$); 23,76 (CH_2); 25,71 (CH_3CHNH); 34,58 (CH_2); 46,71 ($_2(CH_3CH_2)N$); 47,98 ($CH_{3\ (C-2)}$); 52,55 (CH_2N); 99,0 (CH_3CHNH); 119,51 (C-3); 123,51 (C-6); 128,83 (C-5); 129,01 (C-8 & C-7); 148,34 (C-8a); 148,84 (C-2); 159,35 (C-4). APT CH_3: δ 11,40; 20,24; 25,71; 47,98; CH_2: δ 23,76; 34,58; 46,71; 52,55; CH: δ 99,00; 119,11; 123,51; 128,83; 129,01; C: δ 117,35; 148,34; 148,84; 159,35. GC: Rt 10,848.

Compound	Antiviral Activity	Cytotoxicity
	IC_{50} (μg/mL)	TC_{50}(μg/mL)
Chloroquine (3)	10	NC
1	20	NC
2	NA	> 500
4	NA	NC
5	NA	210
6	NA	NC

Table 1. *In vitro* activity of the pyrazolopyridine and quinaldine analogs against coxsakievirus. NA= no activity; NC = no cytotoxicity.

RESULTS

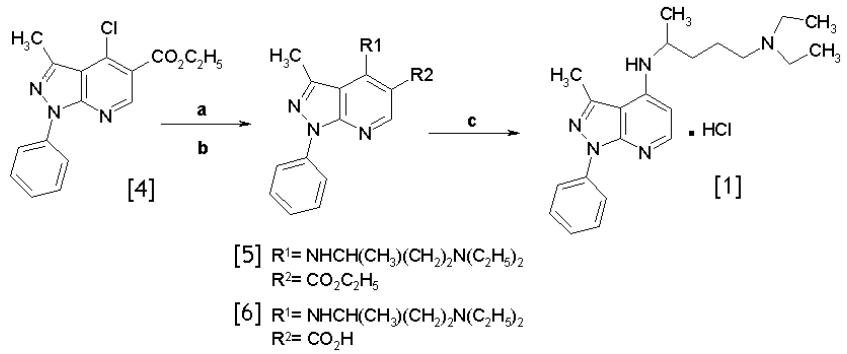
The synthesis of new 1-*H*-pyrazolo[3,4-*b*]pyridine **[1]** was effected exploring the previously described ethyl-5-(4-chloro-3-methyl-1-*H*-phenyl-pyrazolo[3,4-*b*]pyridinyl) carboxylate **[4]** [16,17]. The synthetic route employed uses a nucleophilic substitution reaction of N^1,N^1-diethyl-1,4-pentanediamine on **[4]** to obtain the correspondent amino-ester **[5]**. Basic hydrolysis of the ester moiety furnished the aminoacid derivative **[6]**, which could then be decarboxylated by heating in phosphoric acid to produce the N^4-(3-methyl-1-*H*-phenyl-pyrazolo[3,4-*b*]pyridine)-N^1,N^1-diethyl-1,4-pentanediamine **[1]** (Fig. 2, Scheme 1).

The compound **[1]** showed *in vitro* antiviral activity against coxsakievirus and didn't show cytotoxicity at the concentrations used [18]. In contrast, their synthetic precursors, the [amino-ester **[5]** and amino-acid **[6]** and the compound **[4]** were not active (Table 1).

To investigate if the amine side chain could be an important requirement (pharmacophore moiety) for the antiviral activity showed for chloroquine **[3]** and pyrazolopyridine analog **[1]**, it was prepared another heterocyclic compound with the same side chain **[2]**. The quinaldine analog **[2]** have been prepared just by a halogen-nucleophilic displacement reaction of the N^1,N^1-diethyl-1,4-pentanediamine on 4-chloroquinaldine **[7]** (Fig. 2, Scheme 2).

The structural determination of these compounds showed the signals on ^{1}H and ^{13}CNMR related to the alkyl side chain [19,20]. The analysis of infrared spectrum of the amino-acid derivative **[6]** indicated the presence of absorption at (3500-3300cm^{-1} and at (1580cm^{-1}, attributed to protonate form (NH_2^+,CO_2^-). The mass spectra showed the molecular ions of all the synthetized compounds.

Scheme 1



Scheme 2

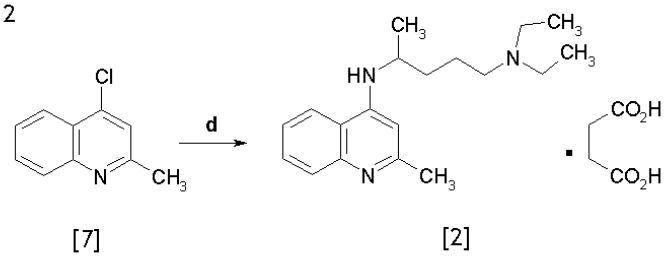


Figure 2.
Scheme 1: synthesis of compound **[1]**.
Scheme 2: synthesis of compound **[2]**.
a) N1,N1-diethyl-1,4-pentanediamine, toluene, reflux, 2h. (41%);
b) 20% v/v NaOH, EtOH, 1h. (60%);
c) 1) H_3PO_4, reflux, 12h;
2) HCl 37% (66%);
d) 1) N^1,N^1-diethyl-1,4-pentanediamine, reflux, 5h;
2) $(CH_2)_2(CO_2H)_2$ (70%).

DISCUSSION

We show that **[1]** inhibits cytophathic effects of the viruses in the coxsackievirus B-3-infected Vero cells at concentration similar to Chloroquine. Chloroquine is a weak base that exerts direct antiviral effects, inhibiting pH-dependent steps of the replication of several viruses [13,21].

Due to similar characteristics of the Chloroquine and the analogues described herein, we could suggest that they act as weak bases, that could affect acidic organells such endosomes. Thereagainst, our studies support the idea that there is something else. Furthermore, the amine side chain presents in Chloroquine **[3]** and pyrazolopyridine analog **[1]** is relevant for the investigated activity, but it was unequaled to quinaldine analog **[2]**. These data show the hypothesis that Chloroquine **[3]** and pyrazolopyridine analog **[1]** probably inhibits viral replication of coxsackievirus B-3 by a different mechanism of inhibition pH-dependent steps.

The theorical considerations of our findings, might also have practical implications especially as additional investigation to therapy for the infections caused by picornaviruses.

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