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Original article

Synthesis and antirhinovirus activity of cyano and amidino substituted flavanoids

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Summary — Cyano and amidino flavans, isoflavans and 3(2H)-isoflavenes were synthesized in order to study their *in vitro* antirhinovirus activity, by comparison with the known corresponding chloro derivatives. The activity of the new compounds was evaluated on rhinovirus 1 B infected HeLa cell cultures by examining their ability to interfere with viral cytopathic effect and with plaque formation. It was found that generally the cyano derivatives behave like the chloro compounds, whereas the amidino derivatives show a lower activity, although always dependent on the position of substituent.

Résumé — Synthèse et activité vis-à-vis du rhinovirus de flavanoides cyano et amidino substitués. *Trois séries de composés (cyano et amidino flavanes, isoflavanes et 3(2H)-isoflavènes) ont été synthétisées et testées pour l'évaluation de leur activité* in vitro *vis-à-vis du rhinovirus 1B, en comparaison avec le 4',6-dichloroflavane connu pour son activité anti-virale. L'activité de ce composé a été étudiée en culture de cellules HeLa infectées par le rhinovirus sérotype 1B, tant pour l'inhibition de l'effet cytopathogène du virus que pour la formation de plaques. Les cyanoflavanes ont présenté une activité significative comparable à celle de dérivés chlorés. Au contraire, les dérivés amidiniques ont montré une activité inhibitrice beaucoup plus faible.*

substituted flavans / substituted isoflavans / substituted isoflavenes / rhinovirus

Introduction

Analogues of 4',6-dichloroflavan (**BW683C**) [1, 2], halogeno derivatives of isoflavan and 3(2H)-isoflavene, were previously prepared by us and tested against rhinovirus 1B (HRV 1B) [3, 4], poliovirus type 2 [5] and hepatitis A virus (HAV) [6]. For the dichloroflavan it was observed [7] that the different activity against various HRV serotypes was in relation to the binding strength of the substance on the viral protein. We noticed a dependence between the molecular shape of the fundamental ring of our halogeno compounds and the activity against different viruses, being the non planarity favourable to obtain antirhinovirus compounds [3].

It was possible that the substitution in the flavan and isoflavan rings of the chlorine atom with the stronger electron-withdrawing cyano group would have improved the binding strength. For instance in the substituted phenoxybenzenes showing antipicornavirus activity, the most effective and broad-spectrum compound is 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile, and generally an electron-poor aromatic ring is necessary for the activity [8].

For different reasons the related amidino compounds could achieve the same effect: the strong basicity of the amidino group would permit stable electrostatic interactions and the capacity to form hydrogen bonds would favour the interaction with viral proteins as well as viral nucleic acids or enzymes. Aromatic amidines are generally potent reversible inhibitors of esteroproteases; some amidino derivatives showed a blocking effect on respiratory syncytial (RS) virus induced cell fusion [9, 10], but no correlation was demonstrated between this activity and their antagonism for tripsin-like proteases [11].

Chemistry

Starting materials for the synthesis were the brominated 3(2H)-isoflavenes (3-phenyl-1(2H)-benzopyrans) (**3a–c**) and flavans (2-phenyl-3,4-dihydro-1(2H)-benzopyrans) (**5a–c**) (fig 1). Among these, only 6-bromo-3(2H)-isoflavene (**3a**) is a new compound; it was prepared by the method described for the synthesis of other halogenated 3(2H)-isoflavenes, among them **3b** and **3c** [3]. From 1-phenyl-2-(2-hydroxymethyl-4-bromophenoxy)ethanone (**1**), obtained by reaction of 2-hydroxy-5-bromobenzylalcohol with phenacyl bromide, the corresponding triphenylphosphonium bromide (**2**) was prepared with triphenylphosphine hydrobromide in boiling acetonitrile. The

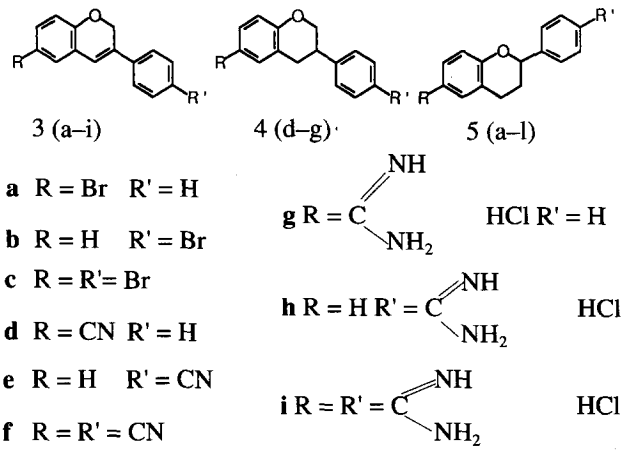

Fig 1. Structures of compounds **3**, **4** and **5**.

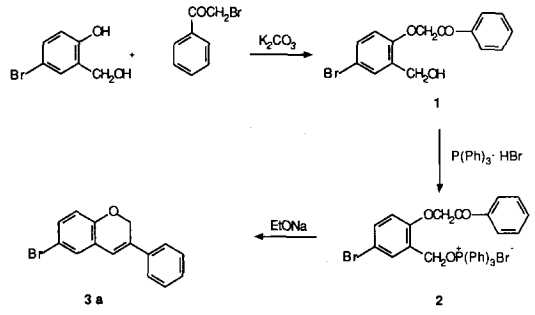

Schéma 1.

successive cyclization was performed with sodium ethoxide in absolute ethanol at room temperature (scheme 1).

The substitution of bromine atom was performed by refluxing compounds **3a–c** and **5a–c** in *N,N*-dimethylformamide with cuprous cyanide. In the case of 4'-substituted isoflavenes (**3b, c**) it was necessary to operate under nitrogen to avoid the formation of oxidation products. 6-Cyano-3(2H)-isoflavene (**3a**) was cited in a patent on heterobicyclic cardioactive aminoacids [12]. The cyanoisoflavans (**4d–f**) were obtained by catalytic hydrogenation of the corresponding isoflavenes (**3d–f**). The amidines (**3g–i, 4g** and **5g–i**) were prepared by Pinner reaction on all the newly synthesized nitriles (**3d–f** and **4d–f**).

Biological results and Discussion

The activity of all the cyano and amidino compounds on HRV 1B multiplication in HeLa (Ohio) cell cultures was studied by examining their ability to interfere with the appearence of virus-induced cytopathic effect (CPE) and plaque formation. Also 6-bromo-3(2H)-isoflavene (**3a**), not tested in the previous report on halogenated-isoflavenes [3], was included in the experiments. **BW683C** was taken as reference compound; under the conditions of the assay the activity of this product was found to be lower than reported [2].

In preliminary experiments, the toxicity on confluent monolayers of uninfected HeLa (Ohio) cells was examined with 5-fold dilutions of each compound, from 200–0.064 μM. Cell toxicity was noted for 200 μM but not for 40 μM solutions; only the cyano-derivatives **3d**, **3f** and **4d** were toxic at the next dilution (40 μM). At non toxic concentration, the viability of cells, as determined by neutral red uptake, was unaffected.

The antiviral activity of compounds against HRV 1B infection was studied by examining the ability of various non-cytotoxic concentrations of each drug to interfere with the appearance of virus-induced CPE and plaque formation. Table I presents the concentrations of the drugs required to inhibit HRV CPE and plaque formation by 50%, indicated as IC_{50} values, as compared with control infected cultures.

Results show that all the drugs tested exhibited an inhibitory effect on HRV multiplication, but only 4',6-dicyanoflavan (**5f**) was more active than the corresponding dichloro compound (**BW683C**). The 2 monosubstituted cyanoflavans (**5d** and **5e**) behaved against rhinovirus in a different manner according to the substitution position; the 6-cyano derivative (**5d**) showed a fairly good activity, whereas the 4'-cyanoflavan (**5e**) was much less active. The occurrence of the opposite situation in the corresponding monoamidino flavans (**5g** and **5h**) may indicate that for a good binding the site of substitution with an electron-withdrawing group is the 6 position, and that for the basic group the 4' position. This observation is in agreement with the good antiviral activity found for 4'-aminoflavan, reported in a patent [13]. Owing to the different experimental conditions in the anti-viral tests, a precise comparison of the results was difficult. If the hydrogen binding also plays a role, our 4'-amidino compound (**5h**) should be more active than 4'-aminoflavan.

Concerning the isoflavene and isoflavan derivatives, the amidines (**3g–h** and **4g**) were less effective in respect to the cyano derivatives (**3d–f** and **4d**), showing activity only at high concentrations. It was noted that in these 2 series, the dicyano compounds (**3f** and **4f**) were less active than monosubstituted derivatives (**3d–e** and **4d–e**) contrary to what was observed for the dicyano flavans and the dihalogenated compounds of the 3 series.

Experimental protocols

Chemistry

Melting points were determined in open glass capillaries on a Büchi SMP-20 apparatus or a Kofler, where indicated, and are

uncorrected. IR spectra were recorded on a Perkin–Elmer 297 instrument; NMR spectra on a Varian EM-390 instrument, using TMS as internal standard. The elemental analyses were within ± 0.4% of theoretical values. 2-Hydroxy-5-bromo-benzyl-alcohol was prepared by reduction of 2-hydroxy-5-bromobenzaldehyde as in reference [14]. Preparation of the bromoflavans **5b** and **5c** has been reported [1]; we prepared these products according to the method used by Jurd to synthesize methoxyflavans [15]. TLC was carried out on aluminium sheets precoated with silica gel 60 F_{254} (Merck) with petroleum ether/ethyl acetate 3:1 as eluent. Spots were observed under ultraviolet light. Column chromatography was performed using silica gel RS, 0.05–0.20 mm (Carlo Erba). The solvents were Carlo Erba RPE–ACS except when otherwise described in the text.

Table I. Action on cytopathic effect (CPE) and plaque formation by HRV 1B in HeLa cell cultures.

Comp	*Maximum non toxic concentr*[a] *(μM)*	IC_{50}[b] *(μM)*	IC_{50}[c] *(μM)*
BW683C	40	0.32	0.038
3a	40	40	5.12
3d	8	8	4.80
3e	40	> 40 (25%)	1.41
3f	8	> 8 (25%)	5.76
3g	40	> 40 (25%)	9.60
3h	40	> 40 (25%)	22.40
3i	40	> 40 (25%)	12.80
4d	8	> 8 (25%)	0.58
4e	40	40	1.47
4f	40	40	14.40
4g	40	40	8.00
5d	40	8	0.14
5e	40	40	2.88
5f	40	0.32	0.023
5g	40	40	8.00
5h	40	8	0.45
5i	40	> 40 (25%)	22.40

[a]Five-fold dilutions of the drugs in the culture medium were applied to the cells over a 72-h period. Four wells were utilized for each concentration of compound. The maximum non toxic concentration was the dilution which did not produce any alteration of cell morphology and viability. The minimum toxic concentration of each drug was, in our experimental conditions, the concentration 5-fold higher than that reported. [b]HeLa cell monolayers in 24-well plates were infected with HRV 1B in order to obtain ≃ 100% CPE 48 h post infection in untreated control wells. Five-fold dilutions of the drugs were added to the medium at the beginning of infection and maintained until the end of the incubation time. Three wells were examined for each drug concentration. The IC_{50} value was the minimal concentration of the drug reducing virus CPE by 50% with respect to the control (100% CPE). When the IC_{50} value was higher than the maximum non toxic concentration the % of inhibition has been reported in parentheses. [c]HeLa cell monolayers in 6-well plates were infected with ≃ 100 plaque forming units (PFU) of HRV 1B with or without the drugs. After infection the cells were overlaid with medium with or without the compounds and incubated until 72 h. Three wells were examined for each drug concentration. The IC_{50} was the concentration of compound reducing the plaque number by 50% and was calculated by plotting the % of plaque reduction *versus* the drug concentration.

2-(2'-Hydroxymethyl-4'-bromophenoxy)-1-phenylethanone ***1***

To a solution of 2-hydroxy-5-bromobenzylalcohol (0.01 mol) and phenacyl bromide (0.01 mol) in dry acetone (25 ml), anhydrous potassium carbonate (0.011 mol) was added. The mixture was heated at 80°C for 3 h while stirring. After cooling, the suspension was filtered and the filtrate evaporated. The crude product was crystallized from ethanol. Mp 77–80°C. Yield 70%. IR (KBr): 3400–3100, 1700 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.05–6.70 (m, 8H, ar); 5.35 (s, 2H, CH_2CO); 4.75 (s, 2H, CH_2O); 3.1–2.9 (bs, 1H, OH). Anal $C_{15}H_{13}BrO_3$ (C, H, Br).

2-Phenacyloxy-5-bromobenzyltriphenylphosphonium bromide ***2***

To a solution of compound **1** (0.01 mol) in acetonitrile (20 ml), triphenylphosphine hydrobromide (0.01 mol) was added. The mixture was heated at 90°C for 2 h while stirring. After cooling, the crystalline precipitate was filtered and washed with ether. More product could be obtained by concentration of mother liquor. Mp 226–31°C. Yield 78%. IR (KBr): 1710 cm^{-1}.

3-Phenyl-6-bromo-1(2H)-benzopyran ***3a***

The crude triphenylphosphonium salt **2** (0.01 mol) was suspended in absolute ethanol (Carlo Erba RPE) (25 ml) and a solution of sodium (0.01 mol) in absolute ethanol was added dropwise at room temperature, while stirring. After 12 h, water was added to the suspension in such amounts so as to have an ethanol–water mixture (3:2). The crude isoflavene **3a** was filtered, washed with water and crystallized from ethanol. Mp 119–22°C. Yield 85%. IR (KBr): 1620, 1590, 1570 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.6–7.2 (m, 7H, H_5, H_7, $H_{2'-6'}$); 6.85–6.7 (m, 2H, H_4, H_8); 5.2 (s, 2H, CH_2). Anal $C_{15}H_{11}BrO$ (C, H, Br).

Synthesis of cyano-3-phenyl-1(2H)-benzopyrans ***3d–f*** *and cyano-2-phenyl-3,4-dihydro-1(2H)-benzopyrans* ***5d–f***

Cuprous cyanide (0.01 or 0.02 mol) was added to a solution of the halocompound (**3a–c** and **5a–c**) [1, 3] (0.01 mol) in *N,N*-dimethylformamide (Carlo Erba RPE with low water content) (250 ml). The mixture was refluxed for a period variable from 24–50 h; for compounds **3b–c** the reaction was carried out under nitrogen. After cooling, the suspension was diluted with water and filtered. The solid was washed with water and extracted with hot ethyl acetate; after evaporation under reduced pressure of the organic solvent, the product was crystallized. Only **3f** had to be first chromatographed on silica gel, eluting with chloroform (table II).

Synthesis of cyano-3-phenyl-3,4-dihydro-1(2H)-benzopyrans ***4d–f***

A solution of the cyanoisoflavenes (**3d–f**) (0.001 mol) in variable amount of ethanol was hydrogenated for 3 h at 45 psi over 0.1 g of Pd/C 5%. After filtration of the catalyst and evaporation of the solvent, the crude product was crystallized (table III).

Table II. Cyano substituted 3-phenyl-1(2H)-benzopyrans (**3d–f**) and 2-phenyl-3,4-dihydro-1(2H)-benzopyrans (**5d–f**).

Compd.	Time (h) reaction	Crystn. solvent	mp (°C)	Yield (%)	Formula	IR (KBr) (cm^{-1})	^1H NMR ($CDCl_3$) (ppm)
3 d	50	Petroleum	127-28	92	$C_{16}H_{11}NO$	2220,1620, 1590,1570.	7.65-7.20 (m, 7H, H_5, H_7, $H_{2'-6'}$); 6.85 (d, 1H, H_8); 6.60 (s, 1H, H_4); 5.25 (s, 2H, CH_2).
3 e	39	Petroleum	111-12	85	$C_{16}H_{11}NO$	2220,1620, 1590,1570.	7.70 (d, 2H, $H_{3'}$, $H_{5'}$); 7.50 (d, 2H, $H_{2'}$, $H_{6'}$); 7.40-6.80 (m, 5H, H_{4-8}); 5.15 (s, 2H, CH_2).
3 f	45	Ethanol	245-47	56	$C_{17}H_{10}N_2O$	2210, 1620, 1600,1575.	7.90-7.20 (m, 6H, H_5, H_7 , $H_{2'}$, $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.05-6.75 (m, 2H, H_4, H_7); 5.30 (s,1H, CH_2).
5 d	25	Petroleum	110-12	92	$C_{16}H_{13}NO$	2210, 1600, 1570.	7.95 (s, 1H, H_5); 7.65-7.20 (m, 6H, H_7, $H_{2'-6'}$); 7.00 (d,1H, H_8); 5.20 (dd, 1H, H_2); 3.10-2.60 (m, 2H, $2H_4$); 2.40-1.90 (m, 2H, $2H_3$).
5 e	24	n-Hexane	105-07	85	$C_{16}H_{13}NO$	2210, 1600, 1570.	7.70 (d, 2H, $H_{3'}$, $H_{5'}$); 7.55 (d, 2H, $H_{2'}$, $H_{6'}$); 7.25-6.80 (m, 4H, H_{5-8}); 5.10 (dd, 1H, H_2); 3.05-2.50 (m, 2H, $2H_4$); 2.30-1.70 (m, 2H, $2H_3$).
5 f	48	Ethanol	156-58	82	$C_{17}H_{12}N_2O$	2200, 1610, 1570.	7.80-7.20 (m, 6H, H_5, H_7, $H_{2'}$ $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.00 (d, 1H, H_8); 5.20 (dd, 1H, H_2); 3.10-2.55 (m, 2H, $2H_4$); 2.45-1.80 (m, 2H, $2H_3$).

Table III. Cyano substituted 3-phenyl-3,4-dihydro-1(2H)-benzopyrans (**4d–f**).

Compd.	EtOH (ml)	Crystn. solvent	mp (°C)	Yield (%)	Formula	IR (KBr) (cm^{-1})	^1H NMR ($CDCl_3$) (ppm)
4 d	100	Petroleum	112-13	93	$C_{16}H_{13}NO$	2210, 1600, 1570.	7.55-7.15 (m, 7H, H_5, H_7, $H_{2'-6'}$); 6.90 (d, 1H, H_8); 4.45 (dd, 1H, H_2, J_{gem} = 10 Hz, J_{2-3} = 3 Hz); 4.10 (dd, 1H, H_2, J_{gem} = 10 Hz, J_{2-3} = 9 Hz); 3.40-2.90 (m, 3H, H_3, $2H_4$).
4 e	200	Ethanol	140-42	92	$C_{16}H_{13}NO$	2200, 1600, 1575.	7.65 (d, 2H, $H_{3'}$, $H_{5'}$); 7.35 (d, 2H, $H_{2'}$, $H_{6'}$); 7.20-6.75 (m, 4H, H_{5-8}); 4.35 (dd, 1H, H_2, J_{gem} = 10 Hz, J_{2-3} = 3 Hz); 4.05 (dd, 1H, H_2, J_{gem} = 10 Hz, J_{2-3} = 9 Hz); 3.50-2.85 (m, 3H, H_3, $2H_4$).
4 f	250	Ethanol	170-74	90	$C_{17}H_{12}N_2O$	2200, 1600, 1570.	8.10-7.20 (m, 6H, H_5, H_7, $H_{2'}$, $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.00 (d, 1H, H_8); 4.50 (dd, 1H, H_2, J_{gem} = 10 Hz, J_{2-3} = 3 Hz); 4.20 (dd, 1H, H_2, J_{gem} = 10 Hz, J_{2-3} = 9 Hz); 3.55-3.00 (m, 3H, H_3, $2H_4$).

*Synthesis of amidines **3g–i** and **5g–i**; **4g***

A solution of the cyanocompound (**3d–f** and **5d–f**; **4d**) (0.01 mol) in either absolute ethanol alone or absolute ethanol/dry ethyl ether (Merck pro analisi) was saturated with hydrochloric acid and left at room temperature. The reaction was followed by TLC until it was completed (average time 7 days). After evaporation of the solvent under reduced pressure, the resulting solid was washed with ethyl ether and solubilized in ammonia-saturated absolute ethanol (≈ 500 ml). After 5 days

at room temperature, the solvent was evaporated under reduced pressure and the residue was washed with ethyl ether and crystallized from ethanol/ethyl ether (table IV).

Material and Methods

Cells and virus

HeLa (Ohio) cells were grown as monolayers in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, antibiotics and glutamine. For cell maintenance the serum concentration was lowered to 2% (maintenance medium).

Rhinovirus 1B was propagated in HeLa cells which were infected at 0.5–1 PFU/cell and incubated at 33°C until an extensive cytopathic effect was recorded. After centrifugation at low speed to remove cellular debris, the infectious supernatant was stored at – 80°C.

Compounds

The drugs were dissolved in absolute ethanol to make stock solutions (1 mg/ml or 0.1 mg/ml) and subsequently diluted with the cell culture medium before use.

Toxicity of compounds for uninfected cells

To determine the cytotoxic effect, confluent monolayers of HeLa cells in 24-well plates were exposed to serial 5-fold dilutions of each drug in maintenance medium for 72 h at 33°C. Cells were inspected daily to detect changes in cell morphology such as swelling, granularity, rounding or floating. The viability of the cells was determined by neutral red uptake at the 3rd day of incubation.

Viral cytopathic effect inhibition assay

For CPE inhibition assay, confluent monolayers of HeLa cells in 24-well plates were infected with HRV 1B (1 h, 33°C) so as

Compd.	mp (°C)	Yield (%)	Formula	IR (KBr) (cm^{-1})	^1H NMR (DMSO d_6) (ppm)
3 g	255-60[a]	45	$C_{16}H_{14}N_2O \cdot HCl \cdot 0.25H_2O$	3200-3000, 1645.	9.65-9.30 (m, 4H, NH_3^+, NH); 7.95-7.40 (m, 7H, H_5, H_7, $H_{2'-6'}$); 7.10 (s, 1H, H_4); 7.05 (d, 1H, H_8); 5.35 (s, 2H, $2H_2$).
3 h	238-41[a]	10	$C_{16}H_{14}N_2O \cdot HCl \cdot 0.4H_2O$	3200-3000, 1670.	9.70-9.00 (m, 4H, NH_3^+, NH); 8.10 (d, 2H, $H_{3'}$, $H_{5'}$); 7.80 (d, 2H, $H_{2'}$, $H_{6'}$); 7.40-6.80 (m, 5H, H_{4-8}); 5.25 (s, 2H, $2H_2$).
3 l	242-45[a]	55	$C_{17}H_{16}N_4O \cdot 2HCl \cdot H_2O$	3350-3000, 1670,1650.	9.75-9.15 (m, 8H, $2NH_3^+$, 2NH); 8.10-7.70 (m, 6H, H_5, H_7, $H_{2'}$, $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.25- 7.05 (m, 2H, H_4, H_8); 5.45 (s, 2H, $2H_2$).
4 g	> 300[a]	38	$C_{16}H_{16}N_2O \cdot HCl$	3250-3000, 1660.	9.60-9.00 (m, 4H, NH_3^+, NH); 8.10-7.75 (m, 2H, H_5, H_7); 7.65-7.25 (m, 5H, $H_{2'-6'}$); 7.00 (d, 1H, H_8); 4.65-4.00 (m, 2H, $2H_2$); 3.55-2.95 (m, 3H, H_3, $2H_4$).
5 g	171-75	18	$C_{16}H_{16}N_2O \cdot HCl \cdot H_2O$	3400-3000, 1670.	9.45-9.15 (m, 4H, NH_3^+, NH); 7.90-7.65 (m, 2H, H_5, H_7); 7.55-7.35 (m, 5H, $H_{2'-6'}$); 7.10 (d, 1H, H_8); 5.30 (dd, 1H, H_2); 2.95-2.75 (m, 2H, $2H_4$); 2.30-1.90 (m, 2H, $2H_3$).
5 h	197-99	37	$C_{16}H_{16}N_2O \cdot HCl \cdot 0.5H_2O$	3350-3000, 1670.	9.80-9.35 (m, 4H, NH_3^+, NH); 8.00 (d, 2H, $H_{3'}$, $H_{5'}$); 7.70 (d, 2H, $H_{2'}$, $H_{6'}$); 7.30-6.75 (m, 4H, H_{5-8}); 5.30 (dd, 1H, H_2); 3.05-2.65 (m, 2H, $2H_4$); 2.45-1.80 (m, 2H, $2H_3$).
5 l	> 300[a]	40	$C_{17}H_{18}N_4O \cdot 2HCl \cdot 1.5H_2O$	3300-3000, 1670.	9.75-9.15 (m, 8H, $2NH_3^+$, 2NH); 8.10-7.60 (m, 6H, H_5, H_7, $H_{2'}$, $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.10 (d, 1H, H_8); 5.40 (dd, 1H, H_2); 3.10-2.65 (m, 2H, $2H_4$); 2.35-1.80 (m, 2H, $2H_3$).

[a]Kofler

to obtain a generalized CPE 36–48 h after infection. The inoculum was decanted, the monolayers were rinsed twice with phosphate-buffered saline (PBS) and added with maintenance medium containing serial 5-fold dilutions of the drugs. The cells were scored microscopically for evidence of CPE each day. Triplicate monolayers were used for each drug concentration.

Plaque assay

The method was essentially as described by Fiala and Kenny [16]. Briefly, monolayers of HeLa cells in 6-well plates were infected for 1 h at 33°C with ≈ 100 plaque forming units (PFU) of virus in the presence or absence of 5-fold dilution of drugs. After washing twice with PBS to remove unadsorbed virus, the monolayers were overlaid with maintenance medium containing 0.75% agarose, 30 mM $MgCl_2$, 15 μM of DEAE–dextran, with or without the corresponding concentration of compounds. After incubation for 72 h at 33°C in 5% CO_2, the cells were stained with neutral red (2 h, 33°C) and the plaques counted. Three wells were utilized for each drug concentration and the mean value of plaque number was calculated. The IC_{50} value was the amount of drug reducing the plaque number by 50% and was calculated by plotting the percentage of plaque reduction with respect to the control plaque count *versus* the compound concentration.

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