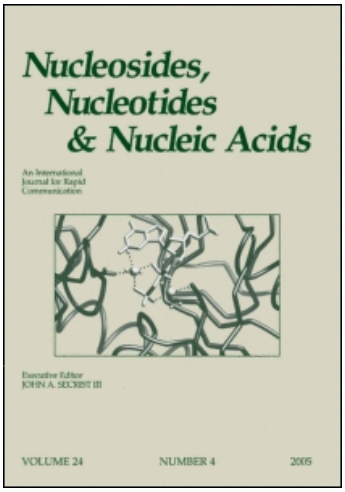

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Relationship Between Conformation and Antiviral Activity -IV. 5-Ethyl-2'-Deoxyurldine and 5-Ethyl-2'-Deoxycytidine

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RELATIONSHIP BETWEEN CONFORMATION AND ANTIVIRAL ACTIVITY - IV. 5- ETHYL-2'-DEOXYURIDINE AND 5-ETHYL-2'-DEOXYCYTIDINE+

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ABSTRACT

5-Ethyl-2'-deoxyuridine (EtdUrd), though a potent inhibitor of herpes simplex virus (HSV) replication, is rapidly catabolized to produce an inactive pyrimidine base by thymidine and/or uridine phosphorylases. 5-Ethyl-2'-deoxycytidine (EtdCyd) was synthesized to confer metabolic stability and thus improve efficacy against systemic HSV infections. EtdCyd was inactive against HSV in the presence or absence of deaminase inhibitors in VERO cells up to 2 mM. The relationship between molecular conformation and antiherpes activity for EtdUrd and EtdCyd is discussed.

INTRODUCTION

The antimetabolite, 5-ethyl-2'-deoxyuridine (EtdUrd) was prepared by Swierkoski and Shugar (1) as an analog of thymidine. EtdUrd was found to be a potent inhibitor of several DNA viruses and have low cytotoxicity against mammalian cells in monolayers (2-7). In contrast, EtdUrd was found to be more toxic when cells are rapidly proliferating (2). Despite the fact that EtdUrd is incorporated into DNA, it has been shown to be non-mutagenic (8,9),

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+For Part III see Gupta *et al.* Nucleosides & Nucleotides. 1995, 14, 1965.

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and non-immunosuppressive (10). The selectivity of EtdUrd against HSV is due to preferential phosphorylation by the virus-encoded thymidine (dThd) kinase [dThd/deoxycytidine (dCyd) kinase] to the 5′-monophosphate (EtdUMP), which is subsequently converted to the triphosphate (EtdUTP) by the thymidylate kinases. Incorporation of EtdUTP into viral DNA by viral DNA polymerase results in inhibition of DNA synthesis and viral progeny formation (11). Clinical studies have shown EtdUrd to be effective in the treatment of herpetic keratitis (12) and genital herpes (13). Despite the fact that EtdUrd is a potent inhibitor of HSV replication, it was ineffective in protecting mice against intracerebral challenge with virus (7) because EtdUrd is rapidly catabolized to an inactive pyrimidine base (5-ethyluracil) by thymidine and/or uridine phosphorylases (14,15). Thus, EtdUrd is of limited usefulness in the treatment of systemic HSV infections.

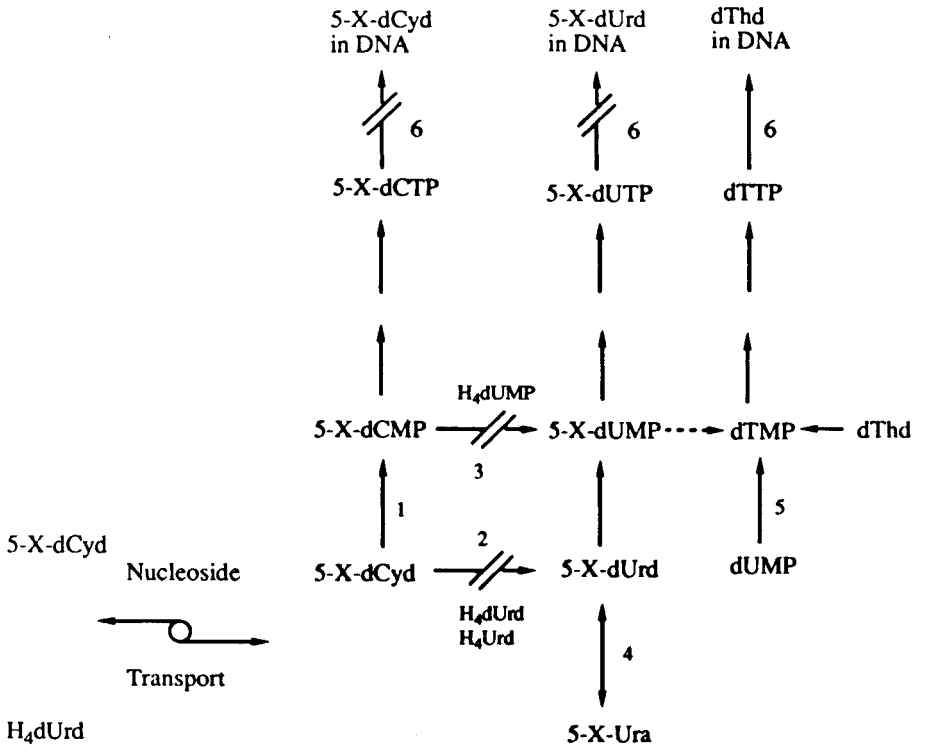
We have hypothesized that it should be possible to overcome the limitations of metabolic instability by the use of deoxycytidine (dCyd) analogs, provided these compounds can be channelled through the dCyd kinase-deoxycytidylate (dCMP) kinase pathway in HSV-infected cells (Fig.1). We have reported that, when deamination was prevented, the antiviral potency of (E)-5-(2-bromovinyl)-2′-deoxycytidine (BrVdCyd) and 5-methoxymethyl-2′-deoxycytidine (MMdCyd) is enhanced (16-19). Halogenated analogs of dCyd are more selective inhibitors of HSV than the corresponding analogs of deoxyuridine compounds (20). This selective action was attributed to the preferential phosphorylation of dCyd analogs by the virus-induced dThd/dCyd kinase (21).

As a part of our research programme on the development of 5-substituted dCyd analogs as inhibitors of HSV, 5-ethyl-2′-deoxycytidine (EtdCyd) was prepared in better yield by a simpler method than synthetic procedures used by Kulikowski and Shugar (22). The activity of EtdCyd alone and in the presence of tetrahydrodeoxyuridine (H_4dUrd) against HSV was assessed and compared with EtdUrd. H_4dUrd is an inhibitor of both Cyd/dCyd deaminase and dCMP deaminase (23). The conformational changes induced by the introduction of an *amino* group at 4 position of the pyrimidine ring and its effect on antiherpes activity are the subject matter of this communication.

RESULTS

Biological Activity

The antiviral activity of BrVdCyd, EtdCyd and EtdUrd against HSV-1 was determined by a plaque reduction assay using RK-13 and VERO cells. BrVdCyd and EtdUrd were



MMdCyd (X = $-CH_2OCH_3$)
BrVdCyd (X = -CH=CH-Br)

FIG. 1 Proposed pathways for metabolism of 5-substituted deoxycytidine analogs in HSV-infected cells. H_4dUrd inhibits both Cyd/dCyd deaminase and dCMP deaminase. Tetrahydrouridine (H_4Urd) inhibits Cyd/dCyd deaminase. 1: dThd/dCyd kinase (virus-induced); 2: dCyd deaminase; 3: dCMP deaminase; 4: dThd and/or Urd phosphorylase; 5: dTMP synthase; 6: DNA polymerase (virus-induced). Inhibition (#).

included as positive controls. EtdCyd had an ED_{50} (concentration required to inhibit cytopathogenicity of HSV-1 by 50%) of > 2 mM compared to BrVdCyd (ED_{50} : 1-2 μM) in VERO cells. Potency of BrVdCyd increased approximately 3-4 fold against HSV-1 (ED_{50} 0.5-0.6μM) in the presence of H_4dUrd; whereas the activity of EtdCyd was not affected (ED_{50}>2 mM). The antiviral potency of BrVdCyd was higher (ED_{50} 0.4-0.5μM) than EtdCyd (ED_{50} ≥ 150 μM) in RK-13 cells. These results are in agreement with values reported for EtdCyd in primary rabbit kidney cells (22) and BrVdCyd in RK-13 cells (16). The concentrations of EtdUrd required to inhibit replication of HSV-1 by 50% in RK-13 and VERO cells was in the range of 8-10 μM. These results are in agreement with earlier findings (2, 22). The activity of EtdUrd was not affected in the presence of H_4dUrd. All compounds were noncytotoxic to RK-13 and VERO cell monolayers up to 512 μM.

Molecular Conformation

The structural formulae for EtdUrd and EtdCyd are shown in Fig. 2. In order to understand why the conversion of EtdUrd into EtdCyd resulted in loss of activity, the two molecules were compared using the computer program PROFIT (24). The stereoscopic representations of the molecules are shown in Fig. 3. The planar pyrimidine ring of EtdCyd was superimposed on EtdUrd and the distances between equivalent atoms were calculated (Table 1). The conformational parameters of the two molecules are compared in Table 2. The bond lengths and angles of EtdCyd (25) are similar to those reported for EtdUrd (26). The conformation about the glycosidic bond, χ [C(2)-N(1)-C(1′)-0(4′)] for both molecules is within the normal range for pyrimidine-2′-deoxyribonucleosides with the *anti* conformation. The values for EtdUrd (236.9°) and EtdCyd (231.0°) are essentially similar for the two molecules. The furanose rings of EtdUrd and EtdCyd have a twist conformation: EtdUrd has C(1′)-*exo*-C(2′)-*endo* puckering and EtdCyd has C(2′)-*endo*-C(3′)-*exo* puckering. The maximum puckering amplitude (τ_m) is the same for both molecules. Comparison of the bond distances between equivalent atoms indicate that the pyrimidine and furanose rings in EtdCyd and EtdUrd are fairly well aligned (Fig. 3, Table 1). The puckering of the furanose ring in EtdCyd is only slightly different from EtdUrd. However, the torsion angle γ [C(3′)-C(4′)-C(5′)-O(5′)] is completely different: t for EtdCyd and g^+ for EtdUrd (Table 2).

NMR Analysis

In order to correlate molecular conformation in solution with biological activity, the conformation of EtdUrd and EtdCyd was also determined by NMR spectroscopy. The proton

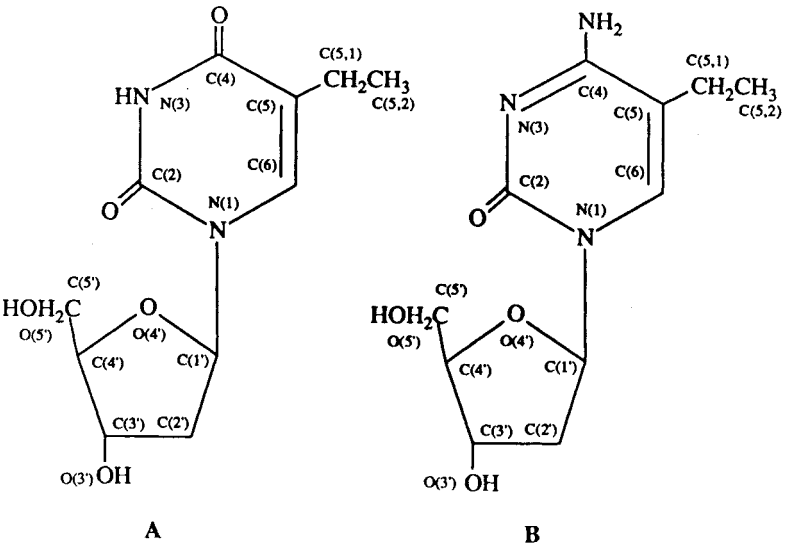


FIG. 2 Structure and atomic numbering of (A) 5-ethyl-2′-deoxyuridine (EtdUrd) and (B) 5-ethyl-2′-deoxycytidine.

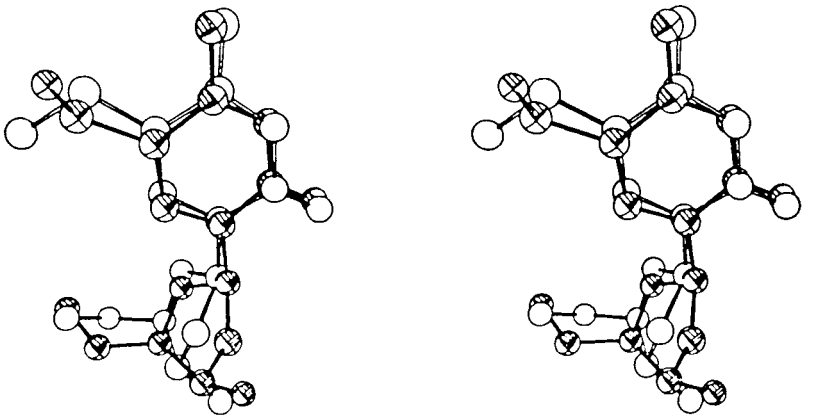


FIG. 3 Stereo view of the superimposed molecules. Hydrogen atoms are omitted for clarity. EtdUrd (open spheres and open bonds); EtdCyd (shaded spheres and solid bonds).

TABLE 1

Comparison of EtdCyd and EtdUrd distances between equivalent atoms when the pyrimidine ring of EtdCyd is superimposed on the pyrimidine ring of EtdUrd.

Atomic Numbering		Distance (Å)
EtdCyd	EtdUrd	
N (1)	N (1)*	0.135
C (2)	C (2)*	0.168
O (2)	O (2)*	0.316
N (3)	N (3)*	0.220
C (4)	C (4)*	0.202
N (4)	O (4)*	0.233
C (5)	C (5)*	0.318
C (5,1)	C (5,1)*	0.564
C (5,2)	C (5,2)*	1.160
C (6)	C (6)*	0.273
C (1′)	C (1′)*	0.273
C (2′)	C (2′)	0.732
C (3′)	C (3′)	0.758
O (3′)	O (3′)	0.598
C (4′)	C (4′)	0.879
C (5′)	C (5′)	1.782
O (5′)	O (5′)	0.279
O (4′)	O (4′)	0.500

* Atoms used in the least square refinement in PROFIT for superimposing.

TABLE 2

Comparison of the conformational parameters of EtdUrd and EtdCyd

	EtdUrd[a]	EtdCyd[b]
χ	236.9° *anti*	231.0° *anti*
Puckering Mode	$^2T_1$ C1′-*exo* C2′-*endo*	^2_3T C2′-*endo* C3′-*exo*
P	140.4	180.3
τ_m	37	38
γ	46.8°(g^+)	174.8°(t)

[a]Ref. 26
[b]Ref. 25

coupling constants and conformational populations are summarized in Tables 3, 4. The conformation of the sugar ring was obtained from the relationship between the proton-proton coupling constants and the pseudo rotational properties of the ring using the computer programme PSEUROT (27). The populations of the three rotamers about the exocyclic C(4′)-C(5′) bond were estimated from the $J_{4'5'}$ and $J_{4'5''}$ coupling constants using the method of Haasnoot *et al.* (28). Both compounds display an approximate 60/40 South/North equilibrium for the deoxyribose conformation. They have a predominant g^+ rotamer for the C(5′) exocyclic side chain. EtdCyd has a higher value for the t rotamer. The populations of the S-state and N-state are similar to the values reported for deoxyribonucleosides (29). Thus, the preferred conformation for EtdCyd and EtdUrd in solution is similar to the crystal structure as determined by X-ray analysis. The syn/anti glycosidic preference for EtdUrd and EtdCyd is shown in Table 5. The glycosidic *syn/anti* equilibrium is biased towards the *anti* position for both compounds. However, it is noticeable that the *anti* glycosidic conformer in EtdCyd has a lower contribution than in EtdUrd (Table 5). In contrast, the X-ray analysis of the solid state shows that the χ angle in both compounds is essentially the same (Table 2).

TABLE 3

Vicinal coupling constants (Hz) of EtdUrd and EtdCyd

310K	EtdUrd		EtdCyd	
37°C	J exp.[a]	J cal.[b]	J exp.[a]	J cal[b]
$J_{1'2'}$	6.9	7.0	6.9	7.0
$J_{1'2''}$	6.2	6.3	6.5	6.5
$J_{2'2''}$	13.8		-14.3	
$J_{2'3'}$	5.9	6.2	6.1	6.3
$J_{2''3'}$	4.4	4.5	4.2	4.5
$J_{3'4'}$	4.0	3.9	4.2	4.0
$J_{4'5'}$	4.1		3.7	
$J_{4'5''}$	3.6		4.4	
$J_{5'5''}$	11.6		-11.7	

[a] J experimental; precision 0.1 Hz
[b] J calculated using PSEUROT with $\tau_m = 36.0^\circ$

TABLE 4

Conformational populations (%): S- and N-conformers of the furanose ring and the three rotamers of the exocyclic C(5′) side chain.

Conformer[a]	S	N	g^{+b}	g^{-b}	t^b
EtdUrd:	62 (160°)[c]	38 (3°)	60	20	20
EtdCyd:	63 (159°)	37 (10°)	55	15	30

[a] In the PSEUROT calculations, τ_m was constrained to 36.0°. The rms deviation for each calculation was: EtdUrd -0.144 E; EtdCyd -0.143 E.
[b] Exocyclic orientation at 37°C. The values reported for the side chain of EtdUrd and EtdCyd are averages over all best fit conformational parameters with a deviation of < 5%.
[c] Numbers in brackets are the calculated pseudorotational angles - P_s and P_n

TABLE 5

Syn/anti glycosidic preference of EtdUrd and EtdCyd

Conformer:	$\eta_6\{1'\}$[a]	$\eta_6\{2'\}$	$\eta_6\{3'\}$	glycosidic preference[b]
EtdUrd:	6	12	5	*anti*
EtdCyd:	5	7	4	*anti*

[a] The common notation used for nOe is ηi [s] which indicates that this is the nOe of nucleus i when nucleus (s) is saturated.

[b] Glycosidic preference refers to a dynamic solution equilibrium that is biased towards either *syn* or *anti*. For *syn* orientations, H_6 is closest to $H_{1'}$ and the nOe to H_6 will be mainly from $H_{1'}$. For *anti* orientations, the nOe to H_6 will be mainly from $H_{2'}$ and $H_{3'}$.

DISCUSSION

The conversion of EtdUrd to EtdCyd resulted in loss of antiherpes activity. These findings were surprising. Previous studies have shown that BrVdCyd and MMdCyd when maintained in the deoxycytidine form are potent and selective inhibitors of HSV-1 replication (16-19). Similarly, 5-iodo-2'-deoxycytidine (IdCyd) is more selective antiherpes agent than the corresponding, 5-iodo-2'-deoxyuridine (20,21).

The conformation of the deoxyribose moiety is important in determining the antiherpes activity of a 2'-deoxynucleoside analog (30-32). The molecular conformation studies by X-ray crystallography (solid state) and NMR spectroscopy (in solution) indicate that the conformation of the 5'-exocyclic side chain [γ angle, O(5') -C(5')-C(4')-C(3')] is important in determining the *activation* of 5-substituted pyrimidine-2'-deoxyribonucleosides by HSV-induced thymidine kinase (HSV-TK). The g^+ conformer (γ about 55°) seems to be the preferred orientation required by HSV-TK; whereas the t conformer (γ about 175°) appears to be an unfavored conformation (30-36). The C(5')-hydroxyl group of EtdUrd has a g^+ rotamer conformation which appears to be the preferred conformation for the viral enzyme. The NMR data agrees to a large extent with the solid state data for EtdUrd. In contrast, for EtdCyd, the exocyclic 5'-OH group has a t conformation in the solid state. However, the NMR data indicates that the C(5') hydroxyl group has a g^+ rotamer conformation. This

anomaly has not been observed in previous studies (30-36) and for the molecule BrVdCyd (37). Thus, one possible explanation for the lack or weak anti-HSV activity of EtdCyd is due to the decreased contribution of the *anti* conformer in the *syn/anti* equilibrium. This results in no phosphorylation of EtdCyd in VERO cells or such a slow rate of phosphorylation in RK-13 cells by the HSV-TK that physiologically significant levels of the triphosphate (EtdCTP), which is the 'active' form of the drug responsible for its antiviral activity, are never reached in infected cells.

In conclusion, results of these investigations indicate that the conformation of deoxyribonucleosides (ligand) should be confirmed by both X-ray and NMR analyses to have a proper understanding of the molecular conformation in relation to biological activity.

EXPERIMENTAL SECTION

5-Ethyl-2′-deoxycytidine and 5-Ethyl-2′-deoxyuridine

The synthesis of EtdCyd was accomplished from EtdUrd in four steps as described previously for MMdCyd (18,35) in better yield than the procedure used previously (22). Hydroxyl groups were protected by acetylation followed by conversion to the triazolyl derivative; which was treated with a saturated solution of ammonia in dioxane to yield 5-ethyl-3′, 5′-diacetyl-2′-deoxycytidine. The diacetyl derivative was purified on a silica gel column using a mobile phase of CH_2Cl_2:MeOH (96:4,V/V). Deblocking and crystallization from MeOH-ether gave EtdCyd as fine white needles (yield 35 %); m.p. 187-188°C; UV (0.1 M HCl), λmax: 287 nm (ϵ, 11900), λmin: 245 nm (ϵ, 700); (0.1 M NaOH), λmax: 278 nm (ϵ, 8000), λmin: 255 nm (ϵ, 4600); ^{1}H NMR (DMSO-d_6): δ, 7.61 (s, 1,6-H), 7.31, 6.80 (broad peaks, 2, 4-NH_2), 6.16 (t, 1, 1′-H), 5.17 (d, 1, 3′-OH), 5.00 (t, 1, 5′-OH), 4.20 (m, 1, 3′-H), 3.75 (m, 1, 4′-H), 3.55 (m, 2, 5′-H, 5′′-H), 2.22 (q, 2, 5-CH_2), 2.04 (m, 2, 2′-H, 2′′-H), 1.03 (t, 3, 5-CH_3). Anal Calcd. for $C_{11}H_{17}N_3O_4$: C 51.76, H 6.71, N 16.46; Found: C 51.58, H 6.87, N 16.36.

5-Ethyl-2′-deoxyuridine was kindly provided by Dr. Derek Ilse, Director, Ortho-Johnson Pharmaceutical Research Institute, Don Mills, Ontario. For antiviral analysis stock solutions were made in phosphate buffered saline (0.1M) and stored at 4°C.

NMR Analysis

The NMR experiments were carried out using a Brucker 300 spectrometer. Spectra were recorded in the Fourier transform mode at 5°, 25°, 37° and 50°. Solutions were made to a concentration of 0.1M in D_2O without adjusting the pD. ^{1}H-NMR spectra were

simulated with the aid of the Brucker routine PANIC and final coupling constants have a precision of 0.1 Hz. Calculations of coupling constants as well as pseudorotational parameters were preformed using PSEUROT (27) assuming a maximum puckering amplitude τ_m= 36.0°. Exocyclic side chain populations were calculated using PANIC experimental coupling constants (28). The *syn/anti* glycosidic torsional preference was deduced from Nuclear Overhauser Effect (nOe) experiments. The enhancements of $H_{1'}$, $H_{2'}$ and $H_{3'}$ on H_6 were recorded and compared. The area under the resonance of the enhanced signal was compared to the area of the same resonance in a control spectrum.

Antiviral Activity

The cell lines used in this study were: African green monkey kidney (VERO), human lung carcinoma (A-549) and rabbit kidney (RK-13). The cells were grown in Eagle's Minimum Essential Medium (MEM) containing 10% foetal bovine serum (FBS) as previously described (2,16). The virus stocks were propagated by low multiplicity of infection of A-549 cell monolayers using HSV-1 (MacIntyre strain) until complete (100%) cytopathic effect and the virus titre was determined according to the procedure of Ayisi *et al.* (2).

The antiviral activity was determined by a plaque reduction assay using agar overlay. Briefly, confluent cell monolayers were infected with 30-50 plaque forming units of virus per well in a 12-well tissue culture plate. Virus dilution were made using serum-free MEM and 0.1 ml was added. The infected cultures were incubated at 37°C. After one hour, the unadsorbed virus was removed by washing with phosphate-buffered-saline (PBS). Each compound dissolved in growth medium at the appropriate concentration was added to each well along with agarose and plates were incubated for 72 hours in a humidified CO_2 (5% atmosphere) chamber. The culture fluid was removed, monolayers fixed, the plaques were stained and enumerated. From dose response curves, the concentration required to reduce the number of plaques by 50% (ED_{50}) was determined. In each experiment, toxicity controls (test compound and medium), cell controls (medium only) and virus controls (virus and medium) were run simultaneously. The assay for each compound was carried out in quadruplicate. The mean values reported are from 4 to 6 determinations.

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