Inhibition of severe acute respiratory syndrome–associated coronavirus (SARSCoV) by calpain inhibitors and β-D-N^4-hydroxycytidine

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We evaluated two types of compounds for efficacy in inhibiting SARSCoV replication *in vitro*: calpain inhibitors (a class of cellular cysteine proteinases) and a number of nucleoside analogues. Cytopathic effect reduction assays visually determined with spectrophotometric verification by neutral red (NR) uptake assay were used to evaluate cytotoxicity and antiviral potency of the compounds. Significantly inhibitory compounds were then evaluated in virus yield reduction assays. Two calpain inhibitors, Val-Leu-CHO (calpain inhibitor VI) and Z-Val-Phe-Ala-CHO (calpain inhibitor III), were the most potent inhibitors of SARSCoV. By virus yield reduction assay, calpain inhibitor VI had a 90% effective concentration (EC_{90}) of 3 µM and calpain inhibitor III had an EC_{90} of 15 µM. β-D-N^4-hydroxycytidine was the most selective nucleoside analogue inhibitor with an EC_{90} of 6 µM by virus yield reduction assay. These compounds or analogues warrant further evaluation as potential therapies for treating SARS or could be used as lead compounds for discovery of more potent SARSCoV inhibitors.

Keywords: severe acute respiratory syndrome-associated coronavirus, SARSCoV, calpain inhibitors, β-D-N^4 -hydroxcytidine

Introduction

Severe acute respiratory syndrome (SARS) is a viral respiratory illness caused by SARS-associated coronavirus (SARSCoV) (Rota *et al*., 2003; Fouchier *et al*., 2003). It is a life-threatening and highly contagious febrile respiratory illness that was initially described in early 2003 in patients from Guangdong Province in southern China. This was quickly followed by numerous cases in Vietnam, Hong Kong, Singapore, Canada and the USA, with some cases in other countries (Hsueh *et al*., 2003).

Treatment for the disease is supportive, as there are no approved or universally recommended therapies for SARS. Initially, systemic corticosteroids were used to suppress the production of the myriad inflammatory mediators that appear in response to the viral infection (Ho *et al*., 2003; Meng *et al*., 2003). Combination therapy with ribavirin and corticosteroids has also been attempted (Peiris *et al*., 2003; Koren *et al*., 2003). However, the efficacy of these treatments has not been demonstrated in controlled studies (Wenzel *et al*., 2003).

Several *in vitro* studies have been undertaken in an attempt to find antiviral agents that suppress SARSCoV replication. Included in the list of agents found inhibitory to the virus have been glycyrrhizin (Cinatl *et al*., 2003a), interferons (Cinatl *et al*., 2003b) and siRNA (Zhang *et al*., 2003; He *et al*., 2003). It has also been suggested that certain products in the armamentarium of Chinese traditional medicine could be of value (Jia & Gao, 2003). In addition, one group of researchers has recommended the use of pentoxifylline because of its selective anti-inflammatory activity and antiviral properties (Bermejo *et al*., 2003).

Because of recent advances in biotechnology, a number of SARSCoV's genes have already been identified and their functions postulated (Snijder *et al*., 2003). Some of these gene products may represent potential targets for antiviral therapy. This list includes SARSCoV polymerase (Xu *et al*., 2003) or replicase (Campanacci *et al*., 2003), an mRNA cap-1 methyltransferase (von Grotthuss *et al*., 2003), cysteine-type proteinases (Yang *et al*., 2003; Chou *et al*., 2003; Theil *et al*., 2003) and, perhaps, several homologues of cellular RNA processing enzymes (Snijder *et al*., 2003). In addition, some have suggested that aminopeptidase N (CD13) might be a putative receptor for the virus and that the interaction between virus glycoprotein and aminopeptidase N could be a target for a small molecule, such as

ubenimix (Kontoyiannis *et al*., 2003). Most recently, Li *et al*. (2003) have shown that angiotensin-convertase enzyme 2 is a functional receptor for SARSCoV. Xiao *et al*. (2003) have demonstrated that the angiotensin-convertase enzyme 2 interacts with defined amino acid sequences of glycoprotein S of SARSCoV. Thus, this interaction between virus glycoprotein and cell receptor represents an additional and partially defined target for antiviral chemotherapy, as well as for vaccine development.

Based on previous studies with other viruses and some of the potential targets described above, we evaluated a number of compounds for inhibitory activity against SARSCoV *in vitro*.

Materials and methods

Chemicals

Calpain inhibitors

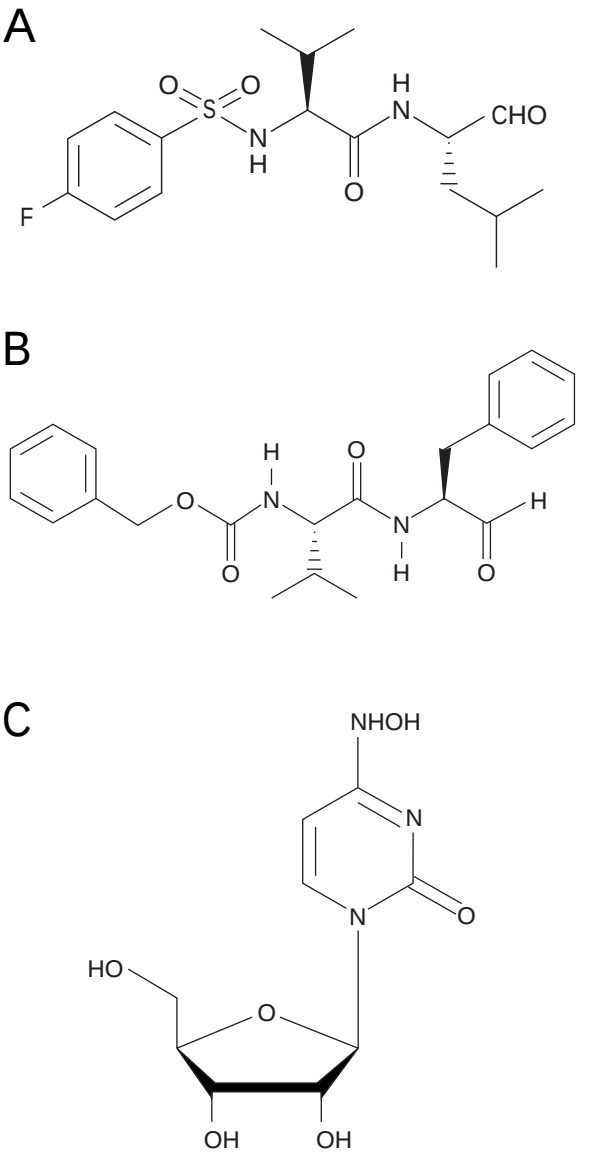
Calpain inhibitors were obtained from CalBiochem-NovaBiochem Corporation (San Diego, Calif., USA) and included *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal (4-fluorophenylsulfonyl-Val-Leu-CHO; calpain inhibitor VI), carbobenzoxy-valinyl-phenylalaninal (Z-Val-Phe-CHO; calpain inhibitor III), *N*-acetyl-Leu-Leu-Nle-CHO (ALLN; calpain inhibitor I), Z-Leu-Leu-Tyr-CH_2F (Z-LLY-FMK; calpain inhibitor IV), benzyloxycarbonylleucyl-norleucinal (calpeptin, Z-Leu-Nle-CHO) and the non-selective calpain inhibitor, 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid. The calpain inhibitor 2, 2S,3S-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64d), was purchased from Sigma-Aldrich (St Louis, Mo., USA).

Nucleosides, nucleotides and nucleoside analogues

N^4-benzoyl-5′-O-(dimethoxytrityl)-5-methyl-2′-O-methylcytidine, N^4-benzoyl-5-O-(dimethoxytrityl)-3′-deoxycytidine, N^4-benzoyl-3′-deoxycytidine, N^4-acetyl-5-methyl-2′-O-methylcytidine, N^4-benzoyl-2′-O-methylcytidine, 2′-deoxycytidine hydrochloride, 2-thiocytidine, 5-bromo-2′-deoxycytidine, 7-deaza –2′-deoxyguanosine and 7-deaza –2′-deoxyadenosine were purchased from Berry & Associates, Inc. (Ann Arbor, Mich., USA). 2′-Deoxycytidine, 3′-deoxycytidine, 2′,3′-dideoxycytidine, cytosine, 2′-deoxyadenosine and 5′-deoxyadenosine were obtained from Sigma-Aldrich; 5′-dideoxyguanosine, chlorocytidine, cyclic cytosine monophosphate (cCMP) and dideoxycytosine triphosphate (ddCTP) were from Axxora LLC (San Diego, Calif., USA); and 2′,3′-deoxyadenosine was from CalBiochem-NovaBiochem Corp.

β-D-N^4-hydroxycytidine was kindly provided by Pharmasset, Inc. (Tucker, Ga., USA) and 3-deazaneplanocin A was donated by Victor E Márquez (National Cancer Institute, Frederick, Md., USA). Cidofovir was obtained from Gilead Sciences (Foster City, Calif., USA). Ribavirin and pyrazofurin were provided by ICN Pharmaceuticals (Costa Mesa, Calif., USA) and human leukocyte derived interferon alpha-n3 (Alferon N Injection®) by Hemispherx Biopharma, Inc. (Philadelphia, Pa., USA).

Figure 1. Structures of the compounds most inhibitory of SARSCoV



A: Calpain inhibitor VI, 4-fluorophenylsulfonyl-Val-Leu-CHO
B: Calpain inhibitor III, Z-Val-Phe-Ala-CHO
C: β-D-N^4-hydroxycytidine

Cell culture studies

Cells and virus

African green monkey kidney cells (Vero 76) were obtained from American Type Culture Collection (ATCC; Manassas, Va., USA). The cells were routinely grown in minimal essential medium (MEM) supplemented with 5% heat-inactivated foetal bovine serum (FBS; Hyclone Laboratories; Logan, UT, USA). For antiviral assays, the serum was reduced to 2% and gentamicin was added to the medium at a final concentration of 50 µg/ml.

SARSCoV, strain Urbani (200300592), was obtained from the CDC and routinely passaged in Vero 76 cells.

Cytopathic effect (CPE) inhibition assay

Barnard *et al*.'s (2001) protocol was used. Compounds were tested at varying concentrations (four 1 $\log_{10}$ or seven 1/2 $\log_{10}$ dilutions) once or twice with this assay and the activity was then verified spectrophotometrically by neutral red (NR) uptake assay on the same plate (see below). Virus [multiplicity of infection (MOI)=0.001] and compound were added in equal volumes to 80–90% confluent cell monolayers in 96-well tissue culture plates. The MOI used was such that 100% of the cells in the virus controls showed cytopathic effects (CPE) within 3–5 days. The plates were incubated at 37°C until the cells in the virus control wells showed complete viral CPE, as observed by light microscopy. Each concentration of drug was assayed for viral CPE inhibition in triplicate and for cytotoxicity in duplicate. Six wells per microplate were set aside as uninfected, untreated cell controls and six wells received virus in medium only per microplate and represented controls for virus replication. Alferon was included as a positive control drug for each set of compounds tested. For all CPE-based assays, the 50% effective concentrations (EC_{50}) were calculated by linear regression analysis of the means of the CPE ratings, expressed as percentages of untreated, uninfected controls for each concentration.

Morphological changes resulting from a compound's cytotoxicity were graded on a scale of 0–5, with 5 being defined as complete cytotoxicity. The 50% cytotoxic doses (IC_{50}) were calculated by regression analysis and a selectivity index (SI) was calculated using the formula: $SI=IC_{50}/EC_{50}$.

Neutral red (NR) uptake assay of CPE inhibition and compound cytotoxicity

This assay was done on the same CPE inhibition test plates described above to verify the inhibitory activity and the cytotoxicity observed by visual observation. The usual correlation between visual and NR assays in our hands has been greater than 95% (Barnard *et al*., 2001). The NR assay was performed using a modified method of Cavenaugh *et al*. (1990) as described by Barnard *et al*. (1999).

Each well of the plate had medium removed and 0.034% NR added. The plate was then incubated for 2 h at 37°C in the dark. The NR solution was removed from the wells, rinsed and the remaining dye extracted using ethanol buffered with Sörenson's citrate buffer. Absorbances at 540 nm/450 nm were read with a microplate reader (Bio-Tek EL 1309; Bio-Tek Instruments, Inc., Winooski, Vt., USA). Absorbance values were expressed as percentages of untreated controls and EC_{50}, IC_{50} and SI values were calculated as earlier described.

Virus yield reduction assay

All compounds with an SI greater than 10 were evaluated in a virus yield reduction assay to confirm the results of the CPE inhibition/NR uptake assays. Infectious virus yields from each well from a second CPE inhibition assay were determined as previously described (Barnard *et al*., 2001). After CPE was scored, each plate was frozen at –80°C and thawed. Sample wells at each compound concentration tested were pooled and titred in Vero cells for infectious virus by CPE assay as described previously by Barnard *et al*. (2002).

A 90% reduction in virus yield was then calculated by linear regression analysis. This represented a 1 $\log_{10}$ inhibition in titre when compared to untreated virus controls.

Evaluation of cytotoxicity in rapidly dividing cells

Cytotoxicity in rapidly dividing cells was evaluated by determining the total number of cells, as reflected by a NR uptake assay after a 3-day exposure to several concentrations of compound (Barnard *et al*., 2002). To quantitate cell growth after 72 h in the presence or absence of drug, the plates were treated as described above for the NR assay. Absorbance values were expressed as percentage of untreated controls and IC_{50} values were calculated by regression analysis.

Results

Using CPE reduction assays, visually assessed and verified spectrophotometrically by NR uptake assay in the same plate, we evaluated a number of metabolic inhibitors, including calpain inhibitors and several nucleotides, nucleosides and nucleoside analogues, for anti-SARSCoV activity in Vero 76 cells. Compounds found active in those two assays were further evaluated for inhibition of infectious virus production.

The most inhibitory calpain inhibitor had the motif Val-Leu-CHO and an EC_{50} of 1 µM as determined by NR assay (Table 1). Calpain inhibitor III (Z-Val-Phe-Ala-CHO) was also active against SARSCoV; the EC_{50}=0.5 µM by visual assay and 1 µM by NR assay.

Of the cytosine-type compounds, β-D-N^4-hydroxycytidine was very active, with an EC_{50} of 5 µM, but with some

cytotoxicity detected, the IC_{50} was equal to 50 µM by NR assay (Table 2). N^4-Benzoyl-5′-O-(dimethoxytrityl)-5-methyl-2′-O-methylcytidine slightly, but selectively, inhibited SARSCoV replication as assessed by NR assay (EC_{50}=4 µM). However, slight viral CPE was evident at every dilution of compound tested (data not shown). N^4-Benzoyl-5-O-(dimethoxytrityl)-3′-deoxycytidine, N^4-benzoyl-3′-deoxycytidine, N^4-acetyl-5-methyl-2′-O-methylcytidine, 2′-deoxycytidine, 3′-deoxycytidine, cytosine, N^4-benzoyl-2′-O-methylcytidine, 2′-deoxycytidine hydrochloride, 2-thiocytidine, 5-bromo-2′-deoxycytidine, 5-chlorocytidine, cidofovir, cyclic cytosine monophosphate (cCMP), dideoxycytosine triphosphate and 2′,3′-dideoxycytidine were inactive.

Of the guansosine-like compounds evaluated, ribavirin was found to be active against SARSCoV in the Vero cells at a very high concentration by NR assay with an EC_{50}=622 µM (Table 3). In this same group of compounds, pyrazofurin was also inhibitory, but only near cytotoxic levels (SI=3 by NR assay). Dideoxyguanosine and 7-deaza–2′-deoxyguanosine were inactive.

None of the adenosine derivatives (2′-deoxyadenosine, 2′,3′-deoxyadenosine, 7-deaza-2′-deoxyadenosine and 3-deazaneplanocin A) was particularly selective in their inhibition of SARSCoV replication, although 7-deaza-2′-deoxyadenosine (2′-deoxytubercidin) was inhibitory at <0.2 µM (Table 4).

The activity of the some of the active compounds was confirmed by a third assay, the virus yield reduction assay (Table 5). The calpain inhibitor, 4-fluorophenylsulfonyl-Val-Leu-CHO, reduced virus yields by 90% at 3 µM, Z-Val-Phe-CHO reduced virus yields by 90% at 10 µM and N-acetyl-Leu-Leu-Nle-CHO had an EC_{90}=15 µM. β-D-N^4-hydroxycytidine reduced virus yields by 90% at 6 µM, which confirmed the activity demonstrated by CPE reduction assay and NR assay.

Discussion

The SARCoV infection induced in Vero 76 cells used in these experiments appeared to provide a reproducible antiviral system for evaluating the test compounds described. Vero cells are standard cells used for many antiviral studies with a number of viruses (i.e., acyclovir and herpes simplex viruses) and, in our experiments, yielded the greatest viral titres. In addition, a very pronounced viral cytopathic effect leading to complete lysis of the cell monolayer was seen in these cells, unlike that seen in Caco-2 cells derived from a human colon adenocarcinoma (See Cinatl *et al*. 2003b). The combined use of visual and NR-determined CPE inhibition, confirmed by virus yield reduction, has been useful in antiviral studies with other viruses as we have described in a number of previous reports (Barnard *et al*., 1999, 2001, 2002). Only one strain of SARSCoV was evaluated in this study. Thus, it remains to be determined if other SARSCoV strains have similar antiviral susceptibility profiles as the Urbani strain.

Table 1. Inhibition of the Urbani strain of SARSCoV replication in African green monkey kidney (Vero 76) cells by calpain inhibitors

Compound	Visual assay			Neutral red assay		
	EC_{50} (µM)	IC_{50} (µM)	SI (µM)	EC_{50} (µM)	IC_{50} (µM)	SI
Selective calpain inhibitors						
4-Fluorophenylsulfonyl-Val-Leu-CHO (VI)	2	>100*	>50	1	>100	>100
Z-Val-Phe-CHO (III)	0.5	20	40	1	10	10
N-Acetyl-Leu-Leu-Nle-CHO (I)	2	20	10	4	11	3
Z-Leu-Leu-Tyr-CH_2F (IV)	2	17	9	7	8	1
Z-Leu-Nle-CHO (I & II)	2	17	9	25	25	1
2S,3S-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (II)	>100	>100	ND	>100	>100	ND
Non-selective calpain inhibitor						
3-(4-Iodophenyl)-2-mercapto-(Z)-2-propenoic acid	>100	>100	ND	>100	>100	ND

*Concentrations higher than those shown were not tested.
ND, not determined.

Table 2. Inhibition of the Urbani strain of SARSCoV replication in African green monkey kidney (Vero 76) cells by cytosine nucleosides and analogues

Compound	Visual assay			Neutral red assay		
	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI
β-D-N^4-hydroxycytidine	10	>100*	>10	5	50	10
N^4-Benzoyl-5′-O-(dimethoxytrityl)-5-methyl-2′-O-methylcytidine	20	25	1	4	23	6
N^4-Benzoyl-5′-O-(dimethoxytrityl)-3′-deoxycytidine	2	2	1	3	3	1
N^4-Benzoyl-3′-deoxycytidine	80	>100	>1	>100	>100	ND
N^4-Acetyl-5-methyl-2′-O-methylcytidine	100	>100	>1	>100	>100	ND
N^4-Benzoyl-2′-O-methylcytidine	>7	>7	ND	>7	>7	ND
Cytosine	>100	>100	ND	>100	>100	ND
2′-Deoxycytidine	>100	>100	ND	>100	>100	ND
3′-Deoxycytidine	>100	>100	ND	>100	>100	ND
2′-Deoxycytidine hydrochloride	>100	>100	ND	>100	>100	ND
2′,3′-dideoxycytidine	>100	>100	ND	>100	>100	ND
Cyclic cytosine monophosphate	>10	>10	ND	>10	>10	ND
Dideoxycytosine triphosphate	>1.5	>1.5	ND	>1.5	>1.5	ND
S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine (cidofovir)	>360	>360	ND	>360	>360	ND
5-Bromo-2′-deoxycytidine	>100	>100	ND	>100	>100	ND
5-Chlorocytidine	>0.25	>0.25	ND	>0.25	>0.25	ND

*Concentrations higher than those shown were not tested.
ND, not determined.

Table 3. Inhibition of the Urbani strain of SARSCoV replication in African green monkey kidney (Vero E6) cells by guanosine nucleosides and analogues

Compound	Visual assay			Neutral red assay		
	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI
Pyrazofurin	8	39	5	8	20	3
Ribavirin	1384	875	ND	622	2293	5
Dideoxyguanosine	>2*	>2	ND	>2	>2	ND
7-Deaza–2′-deoxyguanosine	>100	>100	ND	>100	>100	ND

* Concentrations higher than those shown were not tested.
ND, not determined.

Table 4. Inhibition of the Urbani strain of SARS-CoV replication in African green monkey kidney (Vero E6) cells by adenosine nucleosides and analogue

Compound	Visual assay			Neutral red assay		
	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI
7-Deaza-2′-adenosine	0.2	0.2	1	0.1	0.4	4
2′-Deoxyadenosine	>100*	>100	ND	8	20	3
5′-Deoxyadenosine	>100	>100	ND	>100	>100	ND
2′,3′-Deoxyadenosine	>100	>100	ND	>100	>100	ND
3-Deazaneplanocin A	>380	>380	ND	>380	>380	ND

*Concentrations higher than those shown were not tested.
ND, not determined.

Table 5. Reduction of Urbani strain of SARSCoV infectious virus yields in African green monkey kidney (Vero 76) cells by selected agents

Compound	EC_{50} (μM)	IC_{50}* (μM)	SI
Calpain inhibitors			
4-Fluorophenylsulphonyl-Val-Leu-CHO		3	
>100†	>33		
N-Acetyl-Leu-Leu-Nle-CHO	15	57	4
Z-Val-Phe-Ala-CHO	10	43	4
Nucleoside analogues			
β-D-N^4-hydroxycytidine	6	15	>3
Pyrazofurin	27	32	1

*Determined from a 72 h exposure actively growing to compound.
†Concentrations higher than those shown were not tested.

β-D-N^4-hydroxycytidine was one of the active compounds found in the current study. β-D-N^4-hydroxycytidine has previously been shown to inhibit bovine viral diarrhoea virus and hepatitis C virus (HCV) replicon RNA production in Huh7 cells (Stuyver *et al*., 2003), at concentrations similar to those inhibiting SARS in this study. In cell-free HCV NS5B assays, the compound appeared to act as a weak alternative substrate for the viral polymerase, causing the mobility of the polymerase to change in a gel electrophoresis assay. Thus, it was postulated that β-D-N^4-hydroxycytidine might affect the thermodynamics of the secondary structure of the polymerase to cause inhibition of viral replication. Although there are few proven inhibitors of viral RNA replicases, a small number of other compounds have also been shown to inhibit viral RNA polymerases. The nucleoside analogue 2′-deoxy-2′-fluoroguanosine (2′-fluorodGuo), an influenza virus inhibitor, was shown to be phosphorylated by cellular enzymes and reversibly inhibited influenza virus replication in chick embryo cells (Tisdale *et al*., 1995). In that same study, the triphosphate of 2′-fluorodGuo was a competitive inhibitor of influenza virus transcriptase activity derived from disrupted virus. Recently, Xu *et al*. (2003) described the types of nucleoside analogues that should inhibit the SARSCoV RNA polymerase. The authors suggested that potential nucleoside analogue inhibitors of SARS-CoV RNA-dependent RNA polymerase should contain groups at the 2′ and 3′ positions that are capable of making hydrogen-bonding interactions with the amino acid residues Asp623 and Asn691 of the enzyme. Analysis of the molecular model of SARS-CoV polymerase also suggests that potential nucleoside inhibitors should have the C3′ *endo* sugar puckering conformation in order to maintain its ability to makie a hydrogen bond at the 3′ position and to avoid steric conflicts at the 2′ position. Thus, the molecular model of the SARS RNA polymerase would predict that 2′-C-methyadenosine and 2′-O-methylcytidine could be potential inhibitors of SARS-CoV polymerase. The data presented here showed that a 2′-O-methylcytidine derivative, N^4-Benzoyl-5′-O-(dimethoxytrityl)-5-methyl-2′-O-methylcytidine, was slightly inhibitory to SARSCoV replication. In addition, β-D-N^4-hydroxycytidine seems to partially fulfil some of the criteria for an inhibitor of SARS polymerase described earlier as well.

The 3C-like proteinase or main protease of SARSCoV, designated as $3Cl^{pro}$ or M^{pro} to indicate both its similarity to picornavirus 3C proteinases and that it is the main protease of the virus, can potentially cleave at three unusual cleavage sites with Phe, Met or Val (Anand *et al*., 2003).

Since 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid, a non-competitive, non-peptide inhibitor, was not active, perhaps peptide inhibitors that mimic these sites might selectively and competitively disrupt proteolytic cleavage by the SARSCoV M^{pro}. Interestingly, some of the most active calpain inhibitors contained Val or Phe. However, 4-fluorophenylsulphonyl-Val-Leu-CHO was the most potent and selective inhibitor of the compounds tested, regardless of the assay used. Calpain inhibitor IV and E-64d, both irreversible inhibitors of calpains, inhibited SARSCoV but were not selective inhibitors of the virus, as they were cytotoxic near the same levels viral CPE was inhibited.

These results suggest that the compounds found active in this study should be studied further as potential agents for SARS therapy. More probably, they could be used as lead compounds for the development of even more potent, nontoxic inhibitors of SARSCoV.

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