SARS Coronavirus Anti-Infectives

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Received: May 15, 2005; Accepted: September 18, 2006; Revised: September 19, 2006

Abstract: Severe acute respiratory syndrome (SARS) emerged in late 2002 and was controlled in July 2003 by public health measures. Its causative agent, SARS coronavirus (SARS-CoV) jumped from an animal reservoir to humans and has the potential to re-emerge. Following the sequencing of the genetic code and the deciphering of some of the functions of its proteins, including the cellular receptors and host proteins that participate in the life cycle of the virus, promising lead drugs and new uses of old drugs have been discovered. Patent applications for cathepsin L inhibitors have taken new relevance because of the role of cathepsin L in the entry of SARS-CoV into host cells. Likewise, patent applications for SARS-CoV protease inhibitors and interferon and mismatched dsRNA also need to be watched for potential application in treatment and prevention of SARS-CoV. Here, we review the recent advances and inventions that target SARS-CoV infection in humans.

Keywords: Severe acute respiratory syndrome, SARS, SARS coronavirus, SARS-CoV, anti-infective, anti-viral, main protease, 3CLpro, polymerase, helicase, interferon, interferon-inducer, antibody.

INTRODUCTION

SARS [1-5] is a viral pneumonia with 10% fatality rate caused by a previously unknown coronavirus (CoV) that crossed and adapted to humans from animal reservoirs [6,7]. The disease emerged in late 2002 and had spread to 29 countries within a few weeks, infecting ~8,000 people and causing some 800 fatalities. International cooperation resulted in the dramatic prevention of a potentially catastrophic pandemic [8]. In the 3 years since the epidemic, the molecular evolution [9] of the virus has been worked out and additional novel coronaviruses identified in humans and animals, greatly increasing our knowledge of the Coronaviridae.

The SARS-CoV genome (Fig. **1**) is among the largest in the world of RNA viruses (27-31 kb). It is single-stranded, sense (+), capped and methylated at the 5´ end, and polyadenylated at the 3´ end. SARS-CoV genome has 14 predicted open reading frames (ORF) encoding 28 proteins [10-12]. Through a putative recombination event with an unidentified virus, SARS-CoV acquired a receptor-binding domain (RBD) that is specific for the non-catalytic region of human angiotensin-converting enzyme 2 (ACE2). It also binds civet ACE2 with avidity [13]. After attaching to ACE2, a necessary and sufficient cellular receptor, SARS-CoV spike undergoes conformational change that leads to fusion of its lipid envelope with the cell membrane. The nucleocapsid enters the cytosolic compartment, where cellular translational machinery begins without delay to produce viral replicase enzymes that self-assemble after auto-proteolytic cleavage of the ORF1 gene product. Polyprotein (pp) 1a is translated from ORF1a. Pp1ab is encoded by an overlapping ORF1a and ORF1b, and is translated by a -1 ribosomal frameshift mechanism. Other ORFs and structural proteins are translated from a nested set of 3´-co-terminal subgenomic mRNAs. Spike (S), envelope (E), and membrane (M) proteins are targeted to intracellular membranes between the endoplasmic reticulum and Golgi apparatus. Replicated viral genomic RNA associate with nucleocapsid (N) proteins, which interact with M, triggering viral assembly. This is followed by budding into vesicles, which traffic to the cell surface, where mature virions are released.

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CLASSES OF SARS-COV ANTI-INFECTIVES

More than 30 successful drugs against different viruses are now available, abolishing the notion that viral illnesses cannot be treated specifically. Viral entry, transcription, replication, maturation, and cellular processes usurped by viruses represent possible therapeutic targets. The subject was recently reviewed by De Clercq [14].

However, the challenge now is to be able to respond fast enough to emerging viral diseases as well as to reduce the cost of producing drugs that may only be useful in a small number of subjects - a case of drug therapy competing with public health measures. This quandary was illustrated by the "Katrina-like" emergence and subsidence of SARS-CoV [15]. Table **1** summarizes the various classes of SAR-CoV anti-infectives discussed below.

I. VIRAL ENTRY INHIBITORS

Prevention of viral entry into cells is a conceptually sound antiviral strategy. The "dance" between SARS-CoV and its host cell involves binding, conformational change of S2 and membrane fusion, all of which are targets for therapy.

Chimeric Protein that Neutralizes SARS-CoV Spike Protein

The first step of viral entry is attachment of viral surface molecule with its cellular receptor. Soluble decoy receptors that saturate these viral molecules could be used to prevent viral binding to cells.

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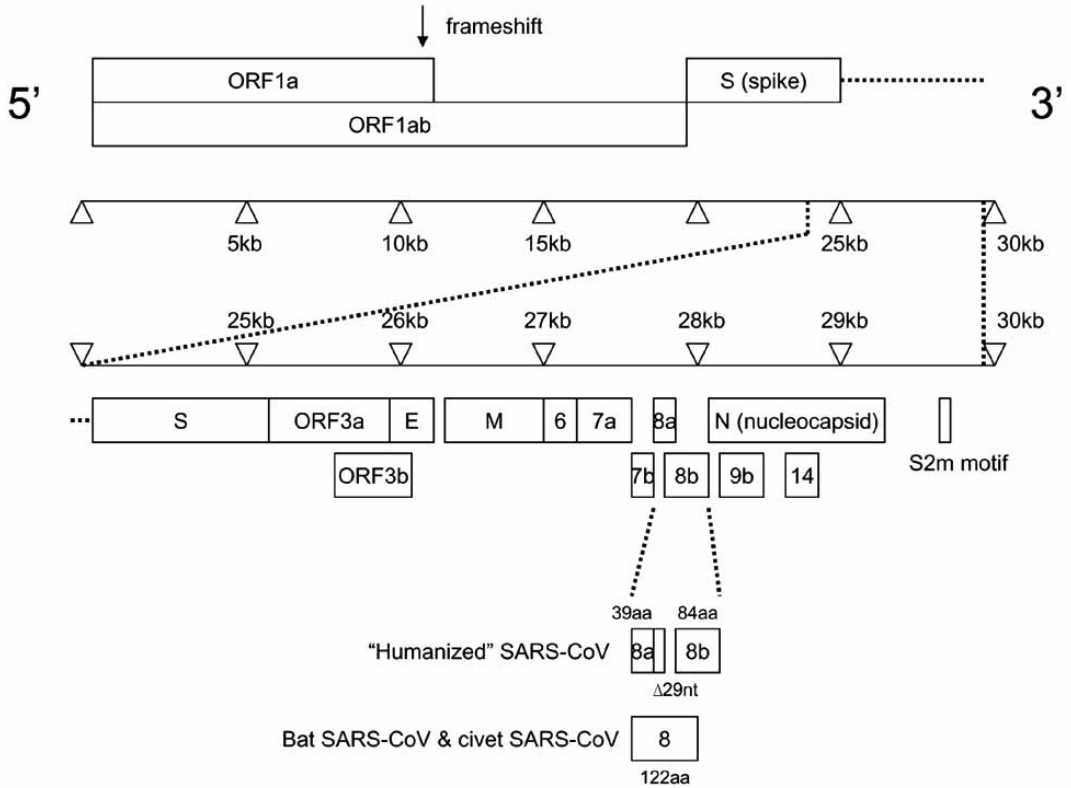

Fig. (1). SARS-CoV genome. A 29-nucleotide stretch is deleted in the humanized strain. Compare with that of bat and civet strains at the bottom.

Table 1. Properties of Some SARS-CoV Anti-Infectives

	Mechanism	IC_{50}[1]/EC_{50}[2]	CC_{50}[3]	SI[4]	Reference
HR121	Heptad repeat (Entry inhibitor)	4.13 μM	-	-	29
HR212	Heptad repeat (Entry inhibitor)	0.95 μM	-	-	29
MDL28170	Cathepsin L inhibitor (Entry inhibitor)	2.5 nM	-	-	40
Chloroquine	Entry inhibitor	8.8 +/- 1.2 μM	261.3 +/- 14.5 μM	30	44
Chloroquine	Entry inhibitor	4.4 +/- 1.0 μM	-	-	45
FP-21399	? Entry inhibitor	Low μM	-	-	53
AG7088	Protease inhibitor	Not effective at 10 μM	-	-	53
KZ7088	Protease inhibitor	-	-	-	54, 55
Ritonavir	Protease inhibitor	Not effective at 50 μM	-	-	53
Saquinavir	Protease inhibitor	Not effective at 50 μM	-	-	53
Lopinavir	Protease inhibitor	50 μM	-	-	53
Lopinavir-like compounds 26-36	Protease inhibitor	23-40 μM	-	-	53

(Table 1) Contd….

	Mechanism	IC_{50}[1]/EC_{50}[2]	CC_{50}[3]	SI[4]	Reference
Lopinavir	Protease inhibitor	1 μg/ml (with ribavirn 6.25 μg/ml)	-	-	67
MAC-5576	Protease inhibitor	05. +/- 0.3 μM	-	-	57
MAC-8120	Protease inhibitor	4.3 +/- 0.5 μM	-	-	57
MAC-13985	Protease inhibitor	7 +/- 2 μM	-	-	57
MAC-22272	Protease inhibitor	2.6 +/- 0.4 μM	-	-	57
MAC-30731	Protease inhibitor	7 +/- 3 μM	-	-	57
Tannic acid	Protease inhibitor	3 μM	-	-	61
TF2B	Protease inhibitor	7 μM	-	-	61
TF3	Protease inhibitor	<10 μM	-	-	61
Hesperetin	Protease inhibitor	8.3 μM	-	-	62
Ribavirin	Polymerase inhibitor	0.5-5 mg/ml	0.2-1 mg/ml	<1	74
β-D-N4-hydroxycytidine	Polymerase inhibitor	5 μM	50 μM	10	78
Aurintricarboxylic acid	Polymerase inhibitor	0.2 mg/ml	37.5 mg/ml	187	83
Valinomycin	?	0.85 μM	68 μM	80	53
Reserpine	?	3.4 μM	25 μM	7.3	53
Reserpine derivatives (compounds 19-24)	?	<100 μM	-	-	53
Aescin	?	6.0 μM	15 μM	2.5	53
Bananins	Helicase inhibitor	<10 μM	>300 μM	>30	88
Glycyrrhizin derivatives (compounds 6, 6, 17 & 18)	?	<100 μM	-	-	53
Glycyrrhizin	?	300 mg/l	>20,000 mg/l	>67	117
Niclosamide	Unknown	1-3 μM	250 μM	-	116
Calpain inhibitor VI	?	3 μM (EC_{90}) virus yield reduction assay	-	-	78
Calpain inhibitor III	?	15 μM (EC_{90}) virus yield reduction assay	-	-	78

1 - IC_{50} – Concentration of a drug that is required for 50% inhibition of viral replication *in vitro*.
2 - EC_{50} – Plasma concentration required for obtaining 50% of the maximal effect *in vivo.*
3 - CC_{50} - Cytotoxic concentration that reduced cell viability to 50%.
4 - SI (Selectivity index) = CC_{50}/EC_{50}.

In HIV infection, more than a decade of research has shown that unmodified decoy receptors are not sufficiently potent [16-18]. The strategy quickly "evolved" into a novel HIV entry inhibitor composed of chimeric proteins, such as CD4-IgG [19-21]. This chimeric molecule has multiple binding regions for HIV-1 gp41 and is currently being tested in human subjects, e.g. ClinicalTrials.gov, identifier NCT 00000876. The chimera overcomes the problems of soluble CD4, such as low neutralizing activity, enhancement of viral infection, and short half-life *in vivo*. Jacobson *et al*. evaluated PRO 542 [22], a CD4-IgG2, in HIV-infected adults in a phase 1 study and reported reductions in plasma HIV RNA and plasma viremia with no dose-limiting toxicities [23]. In another phase 1/2 clinical trial in children with HIV-I infection, PRO 542 was again shown to be well-tolerated besides reducing the viral burden [24].

In a similar fashion, engineered multivalent soluble ACE2 (sACE2)-immunoglobulin might also be efficacious

in neutralizing SARS-CoV [25]. sACE2 can conceivably be improved by using residues 90-93 of civet ACE2 [26].

Membrane Fusion Inhibitors

SARS-CoV shares a similar mechanism with HIV-1 in achieving membrane fusion between virus and host cell. Thus, heptad repeats (HR1 and HR2) located in the S2 domain of SARS-CoV spike protein, oligomerizes to form a six-helix bundle after attachment to ACE2. Spike protein heptad repeat-derived peptides have therefore been predicted [27] and recently shown to inhibit SARS-CoV infection of Vero cells [28]. Further efforts resulted in stable recombinant proteins containing HR1 and HR2, having potent inhibitory activities (HR121 and HR212; IC_{50} values of 4.13 and 0.95 μM, respectively) on entry of the HIV/SARS pseudoviruses [29]. These proteins are also more economical to produce than synthetic peptides. However, they will need to be administered parenterally.

Cathepsin L Inhibitors

Cathepsins are host intracellular enzymes belonging to the papain family of cysteine proteases, of which there are over a dozen types. Most of them are activated by the low pH environments of lysosomes and endosomes, in which they function. Better known cathepsins include type A, required to stabilize sialidase and β-galactosidase; type B, involved in activation of tissue plasminogen and cancer metastasis [30, 31]; type D, involved in mediating apoptosis [32]; type H, expressed in renal oncocytomas but not carcinomas [33]; type K, involved in degradation of type I collagen (mutated in congenital bone disorder) and cancer-induced osteolysis [34]; type L, required for degradation of li in cortical thymic epithelial cells but not marrow-derived antigen-presenting cells [35] and probably participating in malignant transformation [36]; type N, also with collagenolytic activity [37]; and type S, which is inducible by interferon (IFN)-γ in MHC-class II expressing cells and is pivotal in the maturation and peptide-binding competency of class II molecules [38].

To infect cells after SARS-CoV binds to its receptor ACE2, cathepsin L was found to be necessary for an as yet incompletely understood endosomal step [39]. This cellular protease is not however, required by human coronavirus NL63, which also utilizes ACE2 as receptor.

Specific inhibitors of cathepsin L has now been shown to prevent SARS-CoV infection *in vitro* [39, 40]. For example, MDL28170 (calpain inhibitor III) had an IC_{50} of 2.5 nM on substrate cleavage and efficient inhibition of SARS-CoV replication *in vitro*.

Cathepsin L inhibitor, 4-amino-azepan-3-one compounds (Fig. **2**) described in US patent application 20040192674 [41] claims activity in rheumatoid arthritis and prevention of cancer metastasis, among other indications. It would be worthwhile to determine if it possesses anti-viral activity against SARS-CoV.

US20030229226 [42] also teaches small molecule compounds with inhibitory activity against cathepsins K and L.

In addition to small molecules, cellular proteins such as cathepsin L are also susceptible to RNAi-based intervention.

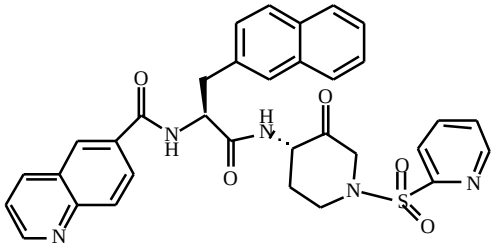

Fig. (2). A representative 4-amino-azepan-3-one compound. Formula A (quinoline-6-carboxylic acid {(*S*)-naphthylen-2-yl-1-[(*S*)-oxo-1-(pyridine-2-sulfonyl)-azepan-4-yl carbamoyl]-ethyl}-amide). It inhibits cathepsin L and may prevent entry of SARS-CoV.

Chloroquine

A potentially useful old drug is Chloroquine (Fig. **3**), being tested for its anti-HIV effect in clinical trials [43]. It is a 9-aminoquinoline discovered by German chemist Hans Andersag in 1934 and used for the treatment of malaria, amebiasis, and autoimmune diseases such as rheumatoid arthritis. It has a high selectivity index of 30 against SARS-CoV in *in vitro* studies [44]. At 10 μM concentration, achievable *in vivo* at dosages used for malaria prophylaxis and treatment, viral inhibition was total by immuno-fluorescence assay [45]. Chloroquine increases endosomal pH, which explains its similar efficacy as ammonium chloride, another lysosomotropic agent, when given up to 5 hours after infection of cell culture by SARS-CoV [44,45]. However, it is also effective when given before viral inoculation onto cell culture, probably due to its interference with terminal glycosylation of ACE2 [44].

Recently, novel synthetic organometallic compounds closely mimicking hydroxychloroquine were found to have selective effect on SARS-CoV. The cytotoxic effects as expected, are less than the parent ferroquine compound [46].

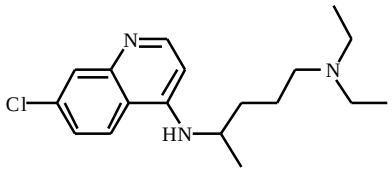

Fig. (3). Chloroquine (7-Chloro-4-(4-Diethylamino-1-Methyl-butylamino) Quinoline). This anti-malarial probably has multiple actions that prevent SARS-CoV from entering cells.

II. SARS-COV PROTEASES AS PROMISING DRUG TARGET

SARS-CoV 3CLpro is a promising drug target because it is essential to the formation of a functional replication complex [47-49]. With the availability of the SARS-CoV genome [10,11,50], a homology model of SARS-CoV chymotrypsin-like protease (main protease, also called 3CLpro) was constructed, providing a basis for the design of

anti-SARS drugs. This model is based on the crystal structures of HCoV-229E main protease (M^{PRO}) and TGEV M^{PRO} in complex with AG7088 [51]. Functional conservation among the coronaviruses suggests that a drug with Gln ↓ (Ser,Ala,Gly) specificity (↓ denotes cleavage site) against SARS 3CLpro may also have activity against the other members [52]. This becomes highly relevant with the recent discovery of several SARS-CoV-like viruses in the wild [6, 7]. This functional conservation was demonstrated by structural homology studies [51].

These results led to the speculation that an anti-rhinoviral drug already in clinical trial, AG7088 (Fig. **4**), might be useful against SARS-CoV. It was subsequently found not to have *in vitro* activity against the virus at a concentration of 10 μM [53]. However, its derivative KZ7088 (Fig. **5**) interacts specifically with the active site of SARS-CoV 3CLpro through six hydrogen bonds [54]. Based on the atomic coordinates obtained by docking KZ7088 with the enzyme's active site, a pharmacophore virtual screening narrowed down the list of compounds worthy of further experiments to 0.03% of the 3.6 million screened [55].

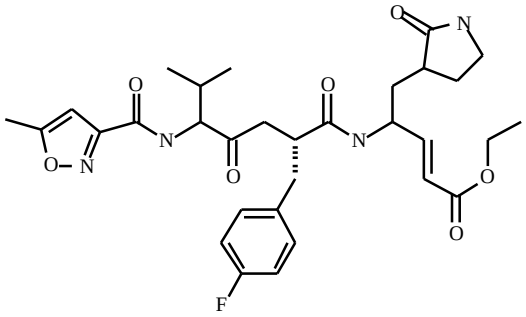

Fig. (4). AG7088. This molecule is being tested in clinical trials against rhinovirus. However, it has no *in vitro* activity against SARS-CoV at a concentration of 10 μM.

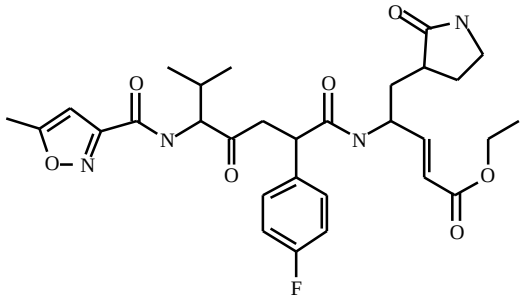

Fig. (5). KZ7088. A derivative of AG7088, KZ7088 interacts specifically with the active site of SARS-CoV 3CLpro.

Recently, proteomic technologies were employed to assist in peptide-based screening of 3CLpro substrate specificity [56]. This information will further assist in drug design. Moreover, this approach using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is readily adaptable to study the substrate specificity of other proteases in a high throughput manner.

Compounds with Activity Against SARS Main Protease (3CLpro)

Using a quenched fluorescence resonance energy transfer assay (Abz-peptide-Nitrotyrosine) and screening 50,000 drug-like small molecules, Canadian scientists discovered five new molecules with 3CLpro IC_{50} in the range of 0.5-7 μM (MAC-5576, -8120, -13985, -22272, -30731) [57].

Recently, benzotriazole esters, intermediates in the synthesis of lopinavir, were found to have k_{inact} (maximal rate of enzyme inactivation) of 0.0011 sec^{-1} and a K_I (inhibitor concentration that supports half the maximal rate of inactivation) of 7.5 nM against 3CLpro [58].

In addition, a group of dicyclic or multi-cyclic compounds with inhibitory activity against SARS-CoV main protease were disclosed in US patent publication 200600-19967 [59]. An example is compound (**18)** (Fig. **6**).

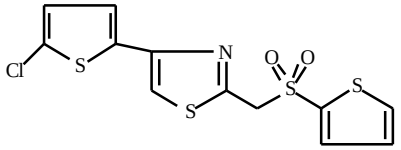

Fig. (6). Compound (18) with inhibitory activity against SARS-CoV 3CLpro (US20060019967).

Natural Substances with Activity Against SARS Main Protease (3CLpro)

Theaflavins from black tea has activity against bovine rotavirus and coronavirus as shown by *in vitro* studies. The EC_{50} against coronavirus was 34.7 micrograms/ml [60].

Substances in black tea with activity against SARS-CoV were discovered as part of a large-scale screening process involving 720 natural products. These include simple and complex oxygen heterocycles, alkaloids, sequiterpenes, diterpenes, pentacyclic triterpenes, and sterols [61]. Assaying for inhibitory activity against 3CLpro proteolytic activity using HPLC, tannic acid and TF2B were identified as having IC_{50} at concentrations <10 μM. Additional experiments using well-known ingredients in tea resulted in the findings that the gallate group-containing TF2B and TF3 have more potent 3CLpro-inhibitory activity than TF1 (Fig. **7**). Thus, tannic acid, TF2B and TF3 join the list of compounds that require evaluation for activity in cell culture.

In another study, *Isatis indigotica* root extract, major compounds from *I. indigotica* root, and several plant-derived phenolic compounds were tested for anti-SARS-CoV 3CLpro inhibitory activity in *in vitro* assays and cell cultures [62]. Cleavage assays with 3CLpro demonstrated that IC_{50} values were in μM ranges for *I. indigotica* root extract, indigo, sinigrin, aloe emodin and hesperetin. Hesperetin (Fig. **8**) dose-dependently inhibited cleavage activity of the 3CLpro, in which the IC_{50} was 8.3 μM in cell-based assay. Thus hesperetin needs to be further investigated.

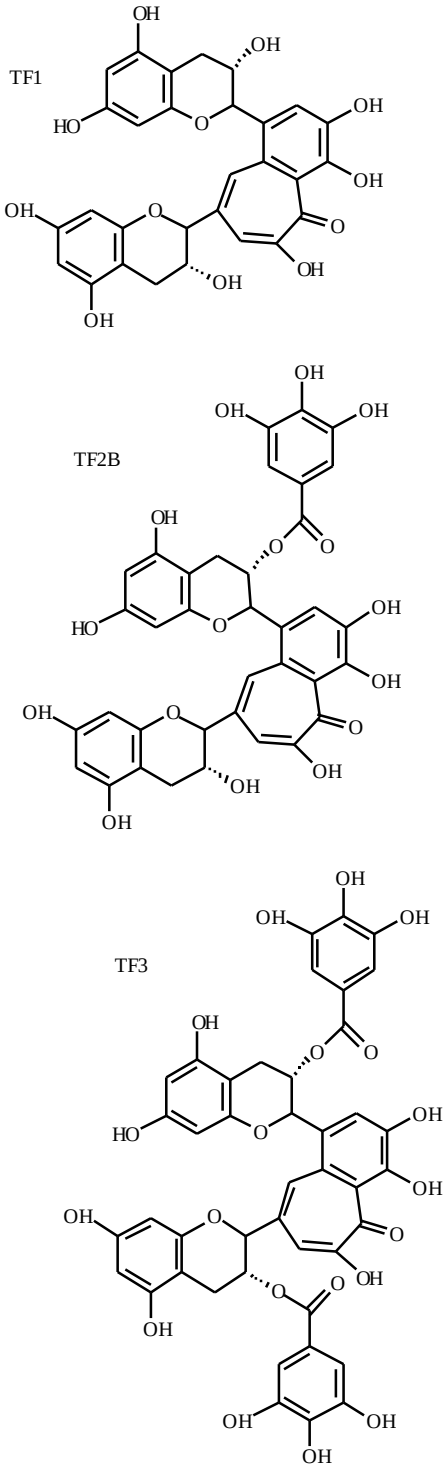

Fig. (7). Chemical structures of TF1, TF2B and TF3.

Papain-Like Cysteine Protease Inhibitors

SARS-CoV Papain-like cysteine protease is a Zn-ribbon-containing proteinase and conserved with the corresponding PL2pro of other coronaviruses. SARS-CoV does not have a homolog of PL1pro that is present in other family members. PL2pro probably took over the function of cleaving the N-terminal portions of pp1a and pp1ab [12,63]. Comparative studies of coronaviral PLpro conservation suggested a link with substrate specificities, which SARS-CoV PL2pro demonstrates. This narrow specificity for substrates is an Achilles' heel that may be exploited for drug development [63].

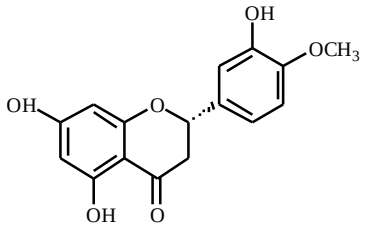

Fig. (8). Hesperetin is a bioflavonoid. It is the aglycone of hesperidin, found in citrus fruits. Hesperetin dose-dependently inhibited cleavage activity of 3CLpro in cell-based assay.

Clinical Experience with Protease Inhibitors

During the epidemic clinicians in Guangzhou observed that HIV-positive patients on HAART appear to be protected against SARS [64,65]. In Hong Kong, the utility of lopinavir-ritonavir was investigated in a multi-center retrospective matched cohort study as initial and rescue therapy for SARS [66]. Patients who received this therapy as initial treatment for SARS had better outcome (reduced death and intubation rate) compared with an uncontrolled group, with lower rate of use of methylprednisolone at a lower mean dose. The results were similar to another report of a subset of those patients treated by the same senior researcher [67]. These clinical trials are in agreement with structural studies that predicted the utility of lopinavir, ritonavir, niclosamide and promazine against 3CL pro [68], with lopinavir and nelfinavir also showing *in vitro* activity [67,69,70].

III. SARS-COV POLYMERASE AS DRUG TARGET

Widespread interest in coronaviruses is relatively recent. As a result, there is little experimental data on the characteristics of coronaviral RNA-dependent RNA polymerase (RdRp) and a consequent lack of inhibitors for this enzyme. The situation is very different for hepatitis B and C, HIV and herpes viruses, where polymerase inhibitors are very successful clinically. SARS-CoV RdRp, which is very important in the viral life cycle, is therefore high on the list of drug targets [71].

Ribavirin

Ribavirin (1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide) was used extensively during the epidemic (Fig. **9**). Derived from D-ribose, it is a long half-life purine nucleoside analog that interacts with viral RNA polymerases, as well as having other activities, such as inhibition of cellular inosinate (IMP) dehydrogenase [72]. In treatment of HCV, ribavirin is thought to act by inhibition of IMP dehydrogenase and by enhancement of Th1 activities [73].

For SARS-CoV however, at a low selectivity index (SI; $SI=CC_{50}/EC_{50}$) of <1 [74], coupled with an absence of demonstrable clinical benefit in uncontrolled series [75,76], the role of ribavirin in treatment is in doubt. The side effects include teratogenicity and a dose-dependent but reversible hemolytic anemia [77]. The N-terminal domain of SARS-CoV nsp14 is homologous to 3'-to-5' exonulcease (ExoN) and may perform RNA proofreading, repair and/or recombination [12]. This unusual capability among RNA viruses may be responsible for the failure of ribavirin in SARS-CoV therapy. Recently, data emerged that ribavirin and other IMP dehydrogenase inhibitors enhance lung infection in a BALB/c mice model [78]. The continued use of ribavirin alone is not recommended [76,77], unless perhaps in combination with an ExoN-inhibitor and supported by experimental results.

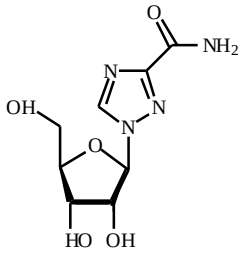

Fig. (9). Ribavirin. Ribavirin has low selectivity index (<1) against SARS-CoV.

Other Nucleotides, Nucleosides, and Nucleoside Analogs

Despite the attractiveness of this group of drugs, β-D-N4-hydroxycytidine (Fig. **10**) is the only nucleoside analog among 26 tested that is selective and has an ED_{90} of 6 μM by virus yield-reduction assay [79]. Its CC_{50} and EC_{50} were 50 and 5 μM, respectively (SI=10). It was earlier found to have selective activity against hepatitis C virus [80-82].

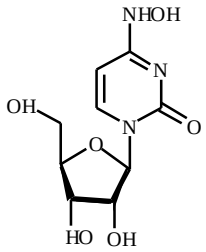

Fig. (10). β-D-N4-hydroxycytidine. This is the only member among 26 nucleoside analogs tested that has selective activity against SARS-CoV.

Other Drugs that Inhibit SARS-CoV Polymerase

Aurintricarboxylic acid (ATA) is a general inhibitor of nucleases found recently to be more potent than IFN-α against SARS-CoV [83]. Molecular docking studies suggest that it inhibits SARS-CoV RdRp by binding to a region in the palm domain (754-766), where two of the three catalytic residues (Asp 760, Asp 761) are located [84].

IV. SARS-COV HELICASE AS DRUG TARGET

This enzyme is another viral enzyme that is worth investigating for drug development. Earlier work has revealed that the viral enzyme unwinds DNA as well as RNA. This property facilitates the development of high-throughput DNA-based helicase assays, which will facilitate the search for inhibitors [63]. That the effort may be worthwhile can be seen in the success of helicase inhibitors currently being developed for herpes viruses [85,86] and HCV [87].

Recently, several bananins (pyridoxal-conjugated trioxa-adamantanes), including iodobananin, bananin (Fig. **11**), eubananin (Fig. **12**) and vanillinbananin (Fig. **13**) were found to non-competitively inhibit the ATPase activity of SARS-CoV helicase with IC_{50} values in the range of 0.5-3 μM [88]. In cell culture, bananin has an EC_{50} of <10 μM and a CC_{50} of >300 μM (SI of >30). Steric hindrance around the pyridoxal ring of some bananins (ansabananin [Fig. **14**] and adenino-bananin) appears to explain why they have no anti-SARS-CoV activity. Surprisingly, bananins may aggravate infection when given prophylactically.

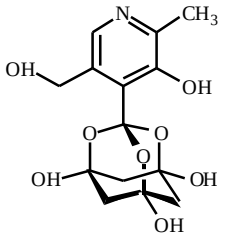

Fig. (11). Bananin.

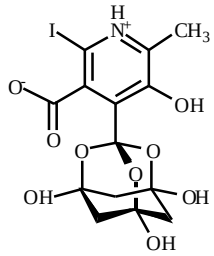

Fig. (12). Eubananin.

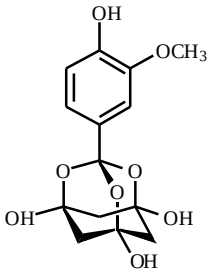

Fig. (13). Vanillinbananin.

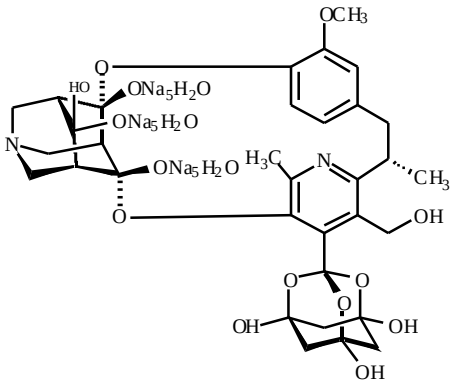

Fig. (14). Ansabananin.

V. INTERFERONS AND INTERFERON INDUCERS

Interferons in SARS

Like most metazoan viruses, SARS-CoV targets the interferon (IFN) signaling pathway [89-91], highlighting the evolutionary importance of IFN against viral infections. Not unexpectedly, evidences of IFN efficacy *in vitro* were readily established during the epidemic.

The type-I IFNs (α/β) but not type-II INF (γ) were found to inhibit SARS-CoV infection and replication [92]. Natural IFN-α and IFN-β have more potent *in vitro* activity than recombinant IFN-γ [93]. Alferon N Injection is the only approved natural, multi-species, alpha-interferon available in the US. *in vitro* Studies demonstrated specific anti-SARS-CoV activity in Vero 76 cell culture. Alferon inhibited SARS-CoV at an EC_{50} of 5,696 +/- 1,703 (SEM) IU/ml (visual) and 10,740 +/- 5,161 (SEM) IU/ml (neutral red). Viral load reduction by one $\log_{10}$ was 78,000 +/- 22,000 (SEM) IU/ml [94].

The *in vivo* activity of interferons were confirmed in macaques, which were protected from SARS-CoV by prophylactic pegylated IFN-α [95]. Postexposure treatment also produced measurable antiviral efficacy, supporting the rationale of employing IFN for prophylaxis or treatment of SARS. In humans, uncontrolled clinical trials have been reported [74,96-100]. No double-blind randomized controlled trial has yet been conducted.

Interferon Inducers

Substances that induce IFN production by dendritic cells and peripheral blood mononuclear cells, such as CpG oligodeoxynucleotide (BW001) yielded supernatant that protected Vero cells from SARS-CoV infection [101].

Hemispherx biopharma recently filed a patent application [94], for treatment of acute and severe viral infections that includes natural human alpha interferons and Ampligen. *in vitro* Cytopathic effect-prevention data on influenzaviruses using various combinations of interferon, ribavirin, oseltamivir and Ampligen were presented.

Ampligen ($rI_n.r(C_{12}U)_n$., Poly APoly U or $rI_nr(C_{29},G)_n$, in which r is ribo) is a mismatched derivative of double-stranded RNAs. It was recently reviewed by De Clercq with the interferons [102]. Mice given Ampligen alone were protected from coxsackie B3 virus-induced myocarditis [103] and flavirus-induced encephalitis [104] but offered only limited protection against lethal pichinde virus challenge [105]. When given with interferon, Ampligen amplifies its effects. It also has synergistic activities with most anti-retrovirals [106] and has generated data in clinical trials [107]. No data is available for SARS-CoV.

VI. MONOCLONAL ANTIBODIES AS ANTIVIRALS

Neutralizing convalescent or engineered antibodies have therapeutic potential in SARS-CoV infection [108]. Neonatal respiratory syncytial virus infections have been prevented by prophylactic administration of MEDI-493, a humanized monoclonal antibody [109].

The SARS animal model ferret, was protected by prior administration of monoclonal antibodies [110]. Moreover, convalescent serum has been used in SARS patients and mice without ill effect, with mice showing measurable antiviral activity against SARS-CoV [111-113].

One group developed an improved B-cell immortalization technique using CpG 2006 (a CpG oligonucleotide) as polyclonal B-cell activator. Employing irradiated allogeneic mononuclear cells, Epstein-Barr virus, and CpG 2006, they interrogated the B-cell memory repertoire of an immune SARS patient. S3.1, a neutralizing antibody from one stable B-cell clone was found to protect mice lungs from SARS-CoV challenge [112]. A total of thirty five monoclonal neutralizing antibodies were isolated in this study. The drawback of the method is that convalescent patients are required.

Another approach uses non-immune human antibody libraries. Eight recombinant human single-chain variable region fragments (scFvs) against the RBD of S protein were identified from a vast library. 80R IgG1, a monoclonal antibody engineered from one such fragment possesses potent neutralizing activity in *in vitro* and animal studies [114,115].

VII. OTHER SARS-COV ANTI-INFECTIVES

Mannose-binding lectin, a component of the innate immune system, proves to play a role in preventing SARS-CoV infection. Deficiency, usually constitutional, is associated with SARS. Therapeutic and prophylactic replenishment needs to be further investigated [116]. Calpain inhibitors have shown some promise, although one of them (calpain inhibitor III) appears to work primarily on cathepsin L rather than the purported main protease 3CLpro [40,79].

Niclosamide

Discovering novel drugs by chemical screening is one strategy but others adopted a strategy of screening old drugs for novel antiviral activities against SARS-CoV [68].

Such efforts resulted in the identification of niclosamide (2',5-dichloro-4'-nitrosalicylanilide) (Fig. **15**), an antihelminthic agent, as having potent activity [68,117]. Vero E6 cells preincubated with drug at 10 μM concentration for 1 hour (also effective 3 hours after infection) and infected by SARS-CoV at an MOI (multiplicity of infection) of 0.1 were

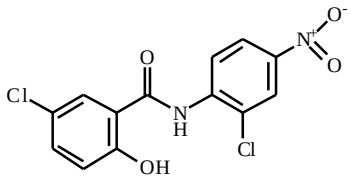

Fig. (15). Niclosamide. This antihelminthic agent has potent *in vitro* activity against SARS-CoV.

observed for protection against CPE (cytopathic effect). Immunofluorescent assay determined the EC_{50} to be between 1 and 3 μM, whereas the CC_{50} (cytotoxic concentration that reduced cell viability to 50%) was 250 μM (at 48 hours incubation). The mechanism of viral inhibition by Niclosamide is not dependent upon inhibition of entry or anti-3 CL^{PRO} activity and remains to be determined.

Glycyrrhizin

Another such compound is glycyrrhizin [118] and its derivatives [53,119]. Glycyrrhizin (Fig. **16**) a triterpenoid saponin found in *Glycyrrhiza glabra* (licorice). It stands out among the inosine monophosphate decarboxylase inhibitors (ribavirin and mycophenolic acid) and orotidine monophosphate decarboxylase inhibitors (6-azauridine and pyrazofurin) as having a high selectivity index ($SI=CC_{50}/EC_{50}$) of 67 against SARS-CoV. Its mechanisms of action are uncertain and may be due to its effects on protein kinase C (cellular signaling pathway), AP-1, NF-κB (transcription factors), and upregulation of inducible nitrous oxide synthase and increased production of nitrous oxide by macrophages. Experimental support of the latter mode of action was provided by the induction of nitrous oxide synthase activity by glycyrrhizin and the fact that addition of nitrous oxide donor (DETA NONOate) inhibits viral replication in Vero E6 cells [118]. Moreover, its effect on lowering plasma membrane fluidity and hence impeding viral entry, is consistent with its observed broad antiviral activity [120]. Side effects include hypertension and hypokalemia in some patients after prolonged treatment.

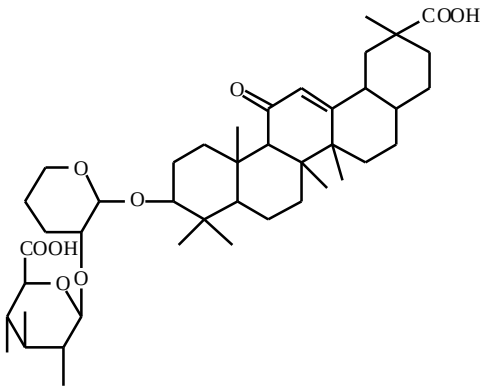

Fig. (16). Glycyrrhizin. Several studies demonstrated a high selectivity index of this agent against SARS-CoV. Its anti-SARS-CoV actions are likely to be multiple.

Valinomycin

Valinomycin (Fig. **17**), a dodecadepsipeptide (a macrocyclic molecule made of twelve alternating amino acids) potassium transporter obtained from the cells of several *Streptomyces* strains, one of them *S. Tsusimaensis*, has EC_{50}, based on ELISA, of 0.85 μM (SI = 80). The mode of antiviral action is unclear [53]. In the same study, FP-21399, and some saponins have also being identified as being highly effective and worthy of further study.

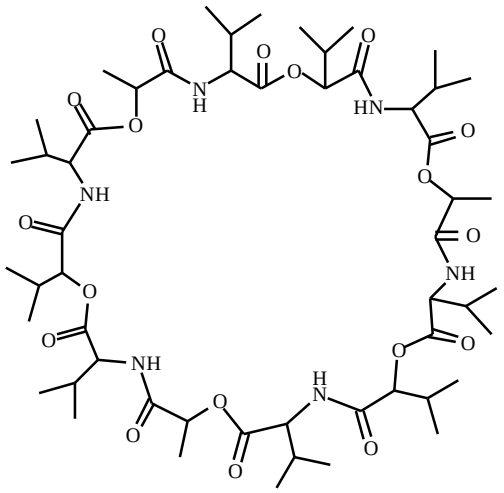

Fig. (17). Valinomycin.

CURRENT & FUTURE DEVELOPMENTS

It is apparent from this survey that anti-SARS-CoV drug development is advancing on many fronts and that many molecules have been screened. It is also apparent that much needs to be elucidated, in particular the *in vivo* activity of these agents in animal studies. However, some of these agents, particularly traditional remedies have well-known safety profiles and should receive priority for further developments [69]. As antivirals come into use, the issue of drug resistance will emerge, as in the case of neuraminidase inhibitor resistance in influenzavirus. Viral escape from drug activity is a virtual certainty and needs to be anticipated and monitored.

Also needing further development are RNA interference-based [121-127] and anti-sense therapies [128], as exemplified by US20060063150 [129], which employs uncharged highly stable morpholino oligonucleotides. The specific delivery of such cargoes into target cells has been a challenge. Recently, Song *et al*. gave us hope that an antibody-mediated delivery via cell-surface receptors may revolutionize this field [130,131].

The rapid accumulation of anti-SARS-CoV medications is a welcome sign of strong basic research. We are now in bad need for paradigm shifts that would lead to more vigorous, but unglamorous, and non-Nobel-winning translational research as well as to inculcate a business ethic of investing in money-losing products that nevertheless might still benefit the pharmaceutical industry in unimaginable ways.

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