Development of Azaindole-Based Frameworks as Potential Antiviral Agents and Their Future Perspectives

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ABSTRACT: The azaindole (AI) framework continues to play a significant role in the design of new antiviral agents. Modulating the position and isosteric replacement of the nitrogen atom of AI analogs notably influences the intrinsic physicochemical properties of lead compounds. The intra- and intermolecular interactions of AI derivatives with host receptors or viral proteins can also be fine-tuned by carefully placing the nitrogen atom in the heterocyclic core. This wide-ranging perspective article focuses on AIs that have considerable utility in drug discovery programs against RNA viruses. The inhibition of influenza A, human immunodeficiency, respiratory syncytial, neurotropic alpha, dengue, ebola, and hepatitis C viruses by AI analogs is extensively reviewed to assess their plausible future potential in antiviral drug discovery. The binding interaction of AIs with the target protein is examined to derive a structural basis for designing new antiviral agents.

INTRODUCTION

The prevalence of fused N-heterocyclic building blocks in pharmaceuticals, functional materials, and natural products suggests their versatility for industrial and innovative applications with promising intellectual property scope.1−8 In this context, azaindole (AI) analogs have been applied as diverse therapeutics due to their antiviral, antibacterial, anticancer, and antiasthma activity and have also been applied against central nervous system (CNS) disorders.9−14 Their widespread therapeutic application is partially due to the unique physicochemical properties of the AI ring. Recently, it was observed that the introduction of a nitrogen atom to replace a −CH group in aromatic and heteroaromatic rings can, under some circumstances, lead to improved physicochemical and pharmacological properties.9 AIs function as H-bond acceptors and donors through their pyridine moiety and pyrrole N−H, respectively, which make them attractive building blocks.15 The different derivatives of azaindoles with diverse substituents at the C2, C3, C4, C5, and C6 positions can be synthesized easily in the laboratory.13 Vemurafenib (1g), venetoclax (1h), peficitinib (1i), and decernotinib (1j), which are Food and Drug Administration (FDA)-approved drugs (Figure 1A), are fair representations of the potential for AIs to be efficacious drugs.

Currently, emerging RNA viruses, such as ebola, influenza, and severe acute respiratory syndrome (SARS) viruses, continue to pose a significant threat to global public health, causing substantial morbidity and mortality, leading to large, unpredictable health care burdens worldwide.16 AIs are prominent heterocyclic motifs in inhibitors of structural and nonstructural proteins from a number of RNA viruses. Intrigued by the development of AI-based frameworks as potential antiviral agents and their future perspective, here we present a critical review spanning the first decade of the 21st century. This review encompasses the physicochemical properties and pharmacological activities of AI analogs against the target proteins of HIV-1, respiratory syncytial virus, dengue virus, ebola virus, hepatitis C virus, neurotrophic alphavirus, and influenza virus to provide an understanding of their application and potential in antiviral drug discovery.

PHYSICOCHEMICAL PROPERTIES THAT ENABLE AIS TO SERVE AS A POTENTIAL TOOL FOR BIOLOGICAL APPLICATIONS

Early investigations of the intrinsic physicochemical properties and in vivo and in vitro pharmacology of unsubstituted AIs have demonstrated their drug-like properties (Figure 1B).1,2,17,18 The four positional isomers of AI analogs, i.e., 4-, 5-, 6-, and 7-AIs, exhibit significantly different pK_a values and lipid–water distribution ratios (D value) (Figure 1A).19,20 The heterobicyclic ring system of AIs consists of a condensed, π-deficient pyridine ring and a π-excessive pyrrole ring.12,13,21

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Incorporating a nitrogen atom into indole to form an AI (1c–f, Figure 1A) confers basic properties to the molecule but reduces the lipid–water distribution ratio compared with that of indole (1a and 1c–f, Figure 1B).19 All 4-, 5-, 6-, and 7-AIs can form cations to different extents at pH 7.4 (1c–f, Figure 1B). More significantly, 5- and 6-AIs exist in their cationic form to an extent of 88% and 78%, respectively, at body pH (1d and 1e, Figure 1B). However, 7-AI exists mostly as a free base at body pH since it exhibits a low degree of ionization (0.15%). The combination of the D value, the pKₐ, and the degree of ionization critically determines the uptake of AIs across biological membranes. The reduced D values of 4-, 5-, 6-, and 7-AIs and 3-, 5-, and 7-triAIs (purine) compared with that of indole depends on both the number and the positions of the nitrogen atoms in the aromatic core (Figure 1A).19 For example, the two nitrogen atoms in 7-AI are in close proximity, leading to a nearly 2-fold reduction in the D value compared with that of indole. However, when the two nitrogen atoms are further apart, the D value drops by nearly one-sixth. The combined effect of the numbers and positions of the nitrogen atoms is reflected in the D value of purine, which is 250-fold lower than that of indole. The chemical stability of AIs is higher than that of indoles under acidic conditions due to protonation of the pyridine nitrogen, which also renders them capable of forming salts. The 4-, 5-, 6-, and 7-AIs can be regarded as excellent bioisosteres of indole or purines that differ only by exchange of a sp² CH fragment(s) by a nitrogen atom(s) and vice versa.12,13,19

The fluorescence properties and proton-transfer tautomerism of AIs have been skillfully applied to form optical probes to investigate protein–protein and protein–nucleic acid interactions.22–29 The use of AIs as optical probes for macromolecular interaction studies provides distinctive advantages over the traditionally used indole analogs. For example, the fluorescence decay of 7-azatryptophan (a 7-AI analog) over most of the pH range is a single exponential when emission is collected over the entire band; however, for tryptophan (an indole analog), a nonexponential fluorescence decay was observed, which made the protein fluorescence more difficult to interpret.30

Thus, 7-azatryptophan was incorporated into synthetic peptides, bacterial proteins, and DNA oligonucleotides as an alternative to tryptophan to study the macromolecular structure and dynamics.31,32 In these studies, 7-AI underwent solvent-assisted excited-state proton transfer (ESPT) catalyzed by alcohols, resulting in an N-7-H tautomer form (Figure 2) that exhibited green emission (≈510 nm).33–36 However, when water was used as the solvent, the N-7-H tautomer-mediated green color emission was not observed due to the slower proton-transfer rate constant (≈10⁹ s⁻¹) as well as dominant radiationless deactivation pathways. One approach to overcome this difficulty was to increase the acidity of the N-1-H form by attaching an electron-withdrawing group such as cyano at C-3, which facilitated the overall ESPT rate in water, thus promoting intensive N-7-H proton-transfer tautomer emission of green light (Figure 2B).33 Alternatively, the pyrrole element was replaced by a pyrazole moiety to form 2,7-diazaindole in which N-2 acted as an efficient electron-withdrawing group to increase the acidity of the N-1-H form (Figure 2C). Thus, 7-AIs and 2,7-diazaindole analogs serve as unprecedented biosensing tools for site-specific water environments in proteins without disrupting their native structure.33

Figure 1. (A) AI-derived, FDA-approved drugs. (B) Comparison of the physicochemical parameters pKₐ (basic), ionization, and lipid–water distribution ratios (D) of indoles, purines, and AIs.
We believe that the intrinsic fluorescence properties of AIs can also be strategically exploited for the detection and fluorescence imaging of viral macromolecules and thus possess diagnostic value.

**HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1) AND AZAINDOLE-BASED INHIBITORS**

HIV belongs to the family Retroviridae, subfamily Orthoretrovirinae, and genus Lentivirus. HIV is classified into HIV-1 and HIV-2 based on genetic characteristics and differences in viral antigens. The genome of HIV-1 consists of two identical, single-stranded RNA molecules enclosed within the virus particle core. HIV-1 contains nine genes, namely, gag, pol, env, tat, rev, nef, vpr, vif, and vpu/vpx, that encode 16 viral proteins that play fundamental roles in the HIV-1 lifecycle. The first three genes, gag, pol, and env, encode structural proteins (matrix, capsid, nucleocapsid, and p6), viral enzymes (protease, reverse transcriptase, and integrase), and env proteins (gp120 and gp41). The rest of the genes encode regulatory proteins (tat and rev) and accessory proteins (nef, vpr, vif, vpu/vpx). vpu is found exclusively in HIV-1, whereas HIV-2 carries vpx. The efficient replication mechanism at different stages of the HIV-1 life cycle is achieved by the physical interaction of pairs of viral proteins. For instance, the HIV-1 envelope glycoprotein gp120 physically interacts with gp41 during viral entry. In addition to HIV pairwise protein interactions, HIV–host protein interactions are known to play essential roles for HIV to take over human cellular systems.

The HIV-1 life cycle consists of approximately nine stages (Figure 3). Briefly, binding of the virus to a cluster of differentiation (CD4) glycoproteins on the host cell surface induces conformational changes in the env trimer that allow the interaction of gp120 with either the CCR5 or the CXCR4 coreceptor, which triggers the next step in the viral fusion process, the rearrangement of gp41. After fusion, the viral RNA is transcribed into linear, double-stranded DNA by HIV-1 reverse transcriptase. The double-stranded DNA is then...
Transported across the nuclear membrane and inserted into the cellular DNA by the enzyme integrase. The integrated HIV-1 provirus serves as a template for the transcription of both viral messenger and genomic RNA by cellular Pol II polymerase. The fully spliced viral RNA, which encodes essential structural and nonstructural proteins, is translated, and the assembly of viral RNA and proteins on the cell surface forms new virions through budding, which are released to infect another host cell (Figure 3). Since the introduction of the first FDA-approved drug zidovudine to treat HIV-1 in 1987, significant progress has been achieved in HIV-1 drug discovery. Currently, 5 nucleoside reverse transcriptase inhibitors (NRTIs), 5 non-nucleoside reverse transcriptase inhibitors (NNRTIs), 6 protease inhibitors (PIs), 1 fusion inhibitor, 1 CCR5 antagonist, 4 integrase inhibitors, 1 attachment inhibitor, 1 pharmacokinetic enhancer, and 24 fixed-dose combinations of one or more drug classes have been approved by the FDA.

The latest addition to the above list, the attachment inhibitor fostemsavir (2), a 6-AI-based prodrug, was approved by the FDA on July 2, 2020 as a therapeutic agent for highly treatment-experienced subjects. Despite the availability of 24 individual drugs (including 2) and 24 fixed-dose combinations, the pursuit of a more effective, mechanistically distinct and noncross-resistant class of drugs to overcome drug toxicity continues in the absence of an effective vaccine, the risk of viral resistance, and the emergence of comorbidities associated with long-term combined antiretroviral therapy (cART).

AI analogs have played a prominent role in the development of HIV-1-attachment inhibitors (Figure 4). The insightful installation of a basic nitrogen atom to replace a CH moiety of...
indole prototypes to form an AI core provided substantial improvements in the molecular, physicochemical, and pharmaceutical properties that translated into better pharmacological and developability profiles. This part of the perspective explores the salient element of the development of Al-based HIV-1-attachment and integrase inhibitors.

**HIV-1-Attachment Inhibitors.** HIV-1 particles infiltrate host cells (T-cells, monocytes, macrophages, and dendritic cells) which express the CD4 glycoprotein receptor to initiate infection. The glycosylated gp120 surface protein of HIV-1 particles physically interacts with gp41 via noncovalent interactions to form the trimeric env spikes. The gp120 protein contains five conserved domains (C1-C5) and five variable domains (V1-V5), which form three key structural regions: the inner domain, the outer domain, and the bridging sheet of the HIV-1 envelope. The gp41 protein contains an N-terminal fusion peptide (FP), two heptad-repeat domains (HR1 and HR2), and a transmembrane anchor (TM). Binding of the HIV-1 envelope protein gp120 to CD4 cells expressed on T-cells triggers an outward domain shift to relieve some of the noncovalent interactions between gp120 and gp41, opening the coreceptor (CCR5 or CXCR4) binding site. Following engagement of the coreceptor, gp120 undergoes further conformational changes that trigger a conformational rearrangement of gp41 to expose the fusion peptide which inserts into the host membrane, leading to fusion of the host cell membrane with the virus and allowing the capsid to enter the cytosol. Each of these discrete steps in the HIV-1-entry process has been verified to be a promising target for anti-HIV-1 drug development. The classification of HIV-1-entry inhibitors depends on their distinctive mechanism of action in the sequential viral entry processes, such as virus attachment (CD4-gp120 interaction), coreceptor binding (CCR5 and CXCR4 inhibitors), and membrane fusion.

Indole glyoxamide 3 (Figure 4), which is an early identified HIV-1 attachment inhibitor (EC_{50} = 153 nM in a pseudotype assay), served as a prototype for many 6-Al-based inhibitors, including the recently FDA-approved produg 2 (Figure 1B). The low in vitro cytotoxicity against a panel of human cell lines, specificity for HIV-1, and synthetic accessibility to allow the introduction of diverse chemical substituents on 3 led to a series of SAR investigations (Figure 4), which collectively contributed to development of the produg, fostemsavir (2, Figure 1B).

The introduction of either F or OCH$_3$ at the C-4 or C-7 position of the indole ring resulted in an increase in antiviral potency (Table 1). The 4-fluoro derivative 4 was observed to be 50-fold more potent than the congener 3 in the pseudotype antiviral assay. The introduction of other substituents, such as Cl, Br, OC$_2$H$_5$, and CN, at C-4 or C-7 also enhanced antiviral potency as compared with 3. The observed increase in the antiviral potency of the disubstituted products 5 and 6 (Table 1) demonstrates the synergistic effect of C-4 and C-7 substitutions. Substitution on C-5 or C-6 and alkylation of the indole nitrogen, however, did not yield an impressive outcome. Furthermore, pharmacokinetic profiling indicated that 3 exhibited modest oral bioavailability ($F_{po} = 29\%$ at a dose of 25 mg per kilogram (mpk)) and high clearance (57 mL/min/kg) in rats. Similarly, 4 (EC_{50} = 2.6 nM in the pseudotype assay) displayed modest bioavailability (17% at a dose of 25 mpk) and clearance (48 mL/min/kg). In contrast, the 4,7-dimethoxy derivative 6 afforded superior oral availability (107%) and low clearance (5.2 mL/min/kg).

**Table 1. HIV-1 Pseudotype Virus Inhibitory Activity, Oral Bioavailability, and Clearance In Vivo of Substituted Indole Glyoxamide Analogs**

| compound no. | substituted indoles | EC\(_{50}\) (nM) | $F_{po}$ (%) in rats | CL (mL/min/kg) |
|--------------|---------------------|-----------------|----------------------|--------------|
| 3            | H H                 | 153             | 29$^b$               | 57$^c$       |
| 4            | F H                 | 2.6             | 17$^b$               | 48$^c$       |
| 5            | F F                 | 0.35            | 11$^c$               | 46.9$^d$    |
| 6            | OCH$_3$ OCH$_3$     | 0.23            | 107$^c$              | 5.2$^d$     |

$^{a}$Dosed as solutions in PEG 400/EtOH (90:10). $^{b}$At 25 mpk. $^{c}$At 5 mpk. $^{d}$At 1 mpk.

Moreover, the potency of the 4,7-dimethoxy derivative was also 5-fold improved over that of the monofluoro analog 4 (Table 1).

Initially, a higher emphasis was placed on optimizing the 4-fluoro derivative 4 based on its promising antiviral profile toward a panel of HIV-1 viruses (Table 2) and its superior oral bioavailability (~100%) in dogs and monkeys dosed at 10 mpk as a solution in PEG 400/ethanol (90:10 v/v). Compound 4 demonstrated good pharmacokinetic properties in the rat and dog following oral dosing as solutions in PEG 400/ethanol. However, the bioavailability of 4 was substantially poorer than that of the solution form when administered as a suspension. Further efforts were made to improve the physicochemical properties of 4 by installing a nitrogen atom and an electron-donating methoxy substituent at the C-7 and C-4 positions, respectively, to create the 7-AI analog 7, which showed a 34-fold improvement in solubility over 4 (Figure 6). The X-ray cocrystal structure data for 7 indicated a mix of H-bonding and hydrophobic interactions with gp120, with the benzoyl group forming parallel and offset alpha-stacking interactions with Phe382 and Trp427 of gp120, respectively. Two hydrogen bonds, one between the backbone N-H group of Trp427 and the

**Table 2. HIV-1-Inhibitory Activity of Compounds 4 and 7 against a Panel of Macrophage and T Cell-Tropic HIV-1 Viruses**

| virus        | 4  | 7  |
|--------------|----|----|
| LA1 (T)      | 2.93 | 2.68 |
| SF-2 (T)     | 62.4 | 26.5 |
| NL4−3 (T)    | 30.8 | 2.94 |
| Bal (M)      | 18.1 | 15.5 |
| SF-162 (M)   | ND  | 3.46 |
| JRFL (M)     | 2.39 | 1.47 |
| TLAV (dual)  | 13.6 | 0.85 |

$^{a}$ND: Not determined. T: Tropic virus that utilizes the CXCR4 coreceptor. M: macrophage-tropic virus that utilizes both the CXCR4 and the CCR5 coreceptors for entry.
Demethylation at the C-4 and C-7 positions, arousing concern over the potential formation of indoloquinolone 11 in vivo. In order to prevent the formation of a reactive quinone, the analogue 10 was designed by introducing a nitrogen atom at the C-6 position of the indole core of 6 (Figure 7).

The oral bioavailability of 10 in rats, dogs, and monkeys after administration as a solution in 90% PEG 400, 10% EtOH was 90%, 57%, and 60%, respectively, whereas 7 exhibited 19%, 77%, and 24% oral bioavailability, respectively, in the same species. Compound 10 demonstrated a clean preclinical safety profile and advanced into phase 1 clinical study. However, in clinical trials, the area under curve (AUC) and C_{max} did not increase proportionally with the administration of increasing dose of compound 10 from 200 to 800 mg. The plasma exposure of 10 showed a 3-fold increase in a solution formulation at a dose of 200 mg, but a 3–5-fold increase was observed when it was administered along with a high-fat meal over the 400–1200 mg dose range. The excessive pill burden and the requirement for dosing with a high-fat meal to achieve targeted plasma exposure posed a challenge that hindered the further development of 10.

A phosphonooxymethyl prodrug approach was designed to improve the solubility of 10 in the gastrointestinal tract (GIT) and to take advantage of the enzyme alkaline phosphatase, which is expressed on the intestinal brush border membrane, to release 10 from the phosphonooxymethyl prodrug 12 by a presystemic cleavage mechanism (Figure 8). Thus, presystemic dephosphorylation of the phosphonooxymethyl prodrug 12, formulated as its (S)-(−)-lysine salt, in the GIT releases the short-lived N-hydroxymethyl derivative 12a, which rapidly liberates one molecule of formaldehyde to produce the parent drug 10. However, a potential concern was the need to avoid precipitation of the parent drug upon dephosphorylation and prior to absorption; thus, balancing the kinetics of phosphate cleavage and the rate of absorption was essential for success. The successful delivery of parent drug 10 is a function of its biopharmaceutical classification system (BCS) class 2 designation based on its experimentally measured low solubility and high intrinsic membrane permeability. The lysine salt of phosphonooxymethyl prodrug 12 demonstrated an improved solubility of >12 mg/mL at pH 5.4 as compared to parent compound 10 which has a solubility of 0.04 mg/mL at pH 4–8. The rapid conversion of 12 into the parent molecule 10 was observed after iv administration to rats, dogs and monkeys.

Figure 5. (A) Improvement in the solubility of 4 upon introduction of a nitrogen atom to replace C-7 and polar methoxy substituents on the indole ring. (B) Two-dimensional interaction diagram of 7 with HIV-1 gp120 (PDB 5U7M).

Figure 6. Active 4-AI analogs 8 and 9 with their potency and cytotoxicity. Here, a mnemonic in which substitution around the attached heteroaromatic ring at C-7 that allows coplanarity with the 4-azaindole was proven to be a useful guide as a predictor of optimal potency within a series.
Carboxamide, sulfonamide, and heteroaryl substituents at fostemsavir (Figure 7) (EC₅₀ = 0.23 nM in pseudotype assay) led to the C-7 position of the indole core which led to the formation of a reactive indoloquinone (EC₅₀ = 0.88 nM (JRFL)).

Figure 7. Installing a nitrogen at C-6 of 6 to form the 6-AI analog 10 to prevent formation of a reactive indoloquinone 11.

The absolute bioavailability of the parent drug 10 after oral administration of 12 was 62%, 93%, and 67% in rats, dogs, and monkeys, respectively. Plasma exposure of 12 increased in a linear manner following administration of 16, 72, and 267 mpk of the prodrug to rats. The AUC and Cmax of 10 were increased up to 3-fold when administered in the prodrug form compared with the administration of the parent drug as a suspension. Similarly, prodrug 12 showed a dose-proportional increase in plasma concentrations at doses ranging from 25 to 800 mg in human clinical studies. Prodrug 12 showed improved clinical potential of 10, further development was abandoned in favor of fostemsavir (2).

Continued SAR studies explored the introduction of carboxamide, sulfonamide, and heteroaryl substituents at the C-7 position of the indole core which led to the identification of compounds with enhanced antiviral potency. Carboxamide 13 showed high human liver microsome stability (HLM) and modest Caco-2 permeability, whereas oxadiazole 14 showed a balanced profile in terms of HLM stability and Caco-2 permeability. However, these substituted indoles were unable to deliver the targeted combination of physicochemical properties and drug-like profiles needed in a clinical candidate (13 and 14, Figure 9B). These findings resulted in the pursuit of the 6-AI core in an effort to overcome the pharmaceutical issues confronted by 10.

In this context, C-7-linked amides and both substituted and unsubstituted heterocycles were installed on the 4-fluoro-6-AI template (Figure 10). The C-linked methylamide 17 (EC₅₀ = 0.09 nM) was suggested to form internal H bonds with the indole NH (amide C=O) and pyridine nitrogen (amide NH) of the 6-AI core, thereby stabilizing a planar conformation. The presence of internal H bonding was corroborated by the X-ray structure of pyrazole 18 (EC₅₀ = 0.07 nM), which revealed H bonding between N-1 of the pyrazole ring and NH of the azaindole to confer coplanarity to this part of the molecule (Figure 10). In the case of oxazole 19 (EC₅₀ = 0.15 nM), despite poor internal H bonding, coplanarity was preserved by means of a positive electrostatic interaction between the O atom of oxazole and the indole NH. However, the oxazole 20 (EC₅₀ = 3.56 nM) and the imidazole 21 (EC₅₀ = 1.3 nM) encountered unfavorable intramolecular interactions (Figure 10) due to electron pair repulsion. Thus, the observed loss of the antiviral potencies of 20 and 21 confirmed a correlation between coplanarity at this region of the molecule and antiviral potency.

Two structural analogs of 6-azaindole (22-23) having 1,2,4-triazole and its 1,2,3-triazole isomer installed at the C-7

Aqueous solubility: >12 ng/mL at pH= 5.4

Aqueous solubility: 0.04 ng/mL at pH= 4-8
The idea to attach C-7, N-linked azoles as a design concept was also explored in the 4-methoxy-substituted 6-AI series (Figure 13) in an effort to overcome the solubility limitations of the 4-fluoro-6-AI series, but the improvement in solubility was modest. The unsubstituted 1,2,4-triazole 25 was characterized by superior antiviral and pharmacokinetic profiles in preclinical species. However, the safety pharmacology data were not encouraging, as 25 inhibited CYP3A4 and the hERG ion channel, arousing concern for potential drug–drug interactions and cardiac liabilities. However, these liabilities were solved with substitution on the 1,2,4-triazole ring of 25 which provided the 3-methyl-substituted analog 26 (also known as tamsavir), which retained the improved pharmacokinetic properties in rats observed with the unsubstituted congener 25.

Prominently, the pharmacokinetic profile of 26 was notably improved over 10 in terms of its low clearance, ~2-fold increased half-life, and 8-fold higher plasma exposure after oral dosing.48,58 In addition, 26 demonstrated acceptable oral bioavailability (52%) when dosed as a suspension in dogs and monkeys. However, 26 showed low crystalline aqueous solubility (0.022 mg/mL) but high membrane permeability. These characteristics of 26 placed this molecule as a BCS class 2. The tris(hydroxymethyl)aminomethane salt of the phosphonoxyethyl derivative of 26, known as fostemsavir 2 (Figure 12), was prepared as a prodrug. It exhibited >11 mg/mL aqueous solubility and 80–122% oral bioavailability for the parent drug 26 in rats, dogs, and monkeys.48,58 At lower doses (≤25 mg), the AUCs of both 26 and 2 displayed similar trends in rats and dogs; however, at 200 mg/kg, administration of the prodrug 2 demonstrated superior plasma exposure of the parent drug compared with that of 26.

Comprehensive biochemical profiling demonstrated that attachment inhibitors based on 6-AIs bind to HIV-1 gp120 and interfere with its attachment to the CD4 receptor.50,58 Another mechanism has been proposed in which 6-AIs hinder the exposure of gp41 following CD4 and coreceptor-induced conformational changes by forming a ternary complex with gp41 and gp120. The binding mode of 26 to gp120 from X-ray cocrystal studies indicated that the benzoyl group of 26 engages in parallel π-stacking interactions with Trp427 in the

Figure 9. (A) General structure of 4-fluoro/methoxy indole glyoxamides. (B) C-7-Substituted 4-fluoroindole glyoxamides with the antiviral potency, microsomal stability, membrane permeability, and oral bioavailability of 13 and 14 presented, and structures of potent HIV-1 inhibitors 15 (methylated thiazole) and 16 (thiazole).
inner and outer interface domains of the β20–β21 loop of gp120 (Figure 13). Two H bonds, first between the backbone NH moiety of Trp427 and the oxoacetamide C=O of 26 and second between the azaindolic N–H group and the side chain carboxylate group of Asp113, have been observed. In addition to the above, compound 26 binds with gp120 through

Table 3. Comparison of Antiviral and Rat Pharmacokinetic Profiles of 10, 22, and 23

| Compound No. | JRFL virus EC50 (nM) | AUC, 24 h (μM·h) | Cmax (nM) | CL, iv (mL·min⁻¹·kg⁻¹) | F (%) | HuPB (%) |
|--------------|----------------------|-------------------|-----------|-------------------------|-------|----------|
| 10           | 0.88                 | 6.3 ± 2.7         | 1889 ± 647| 13 ± 4.0                | 90    | -        |
| 22           | 0.07                 | 83.7 ± 9.8        | 8764 ± 810| 0.7 ± 0.12              | 95    | 97       |
| 23           | 0.05                 | 86 ± 33           | 9484 ± 279| 1.6 ± 0.2               | 64    | 95       |

*aDosed as solutions in PEG 400/EtOH (90:10 v/v). bOral Cmax and AUC adjusted to 5 mg/kg. cHuman plasma binding.

Figure 10. Suggested favorable and unfavorable intramolecular interactions exemplified by C-7-substituted, 4-fluoro-6-AI analogs.
hydrophobic interactions with Trp112, Asp113, Leu116, Thr202, Val255, Phe382, Ile424, Met426, Trp427, Gln432, Thr202, Val255, Phe382, Ile424, Met426, Trw427, Gln432, Met434, and Met475. The analysis of the cocrystal structure of 26 with HIV-1 env revealed that 26 does not allow CD4 to bind to gp120, which is necessary for conversion of the prefusion conformation of the env trimer to the postfusion conformation. At low concentration, 26 stabilizes a prefusion conformation of the env trimer and interferes with the env conformational changes induced by the CD4, whereas at higher concentration 26 binds at the allosteric site in such a manner that it does not allow CD4 attachment on gp120. Figure 14A provides the summary of the structure-guided optimization pathway along with a brief synopsis on the important outcome of each pathway presented. Figure 14B presents important essential structural features for AI-based attachment inhibitors.

The application of a bioconjugation strategy to generate chemically programmed antibodies by covalently attaching the entry inhibitors 7 and 10 to monoclonal antibody (mAb) 38C2 via an N-acyl-β-lactam-derived linker has been described. This study was focused on identifying an AI–antibody conjugate that would recognize gp120 by targeting the conserved attachment inhibitor binding site. It was hypothesized that the covalent conjugation of a mAb 38C2 (aldolase antibody) with gp120 inhibitors will improve the pharmacokinetics profile of the inhibitors. Accordingly, N-acyl-β-lactam derivatives 31 and 32 were synthesized using inhibitors 7 and 10 and then covalently linked to mAb 38C2 to afford chemically programmed antibodies 33 and 34 (Figure 15). Subsequently, the conjugated bivalent antibodies 33 and 34 and their corresponding N-acyl-β-lactam derivatives 31 and 32 were advanced into virus neutralization assays using U87.CD4.CCR5 cells transfected with HIV-1 infectious virus (JRFL) to monitor their antiviral efficacy. Compounds 31 and 32 demonstrated IC50 values of >200 and 67.50 nM, respectively. The conjugated antibodies 33 and 34 exhibited IC50 values of >1000 and 128.6 nM, respectively.

The weak neutralization activities of 31 and 33 were consistent with low gp120 binding due to the C-4 substitution, which disrupted JRFL–gp120 binding, as reported earlier. The significant IC50 values of 32 and 34 indicates that the linker attachment at the C-7 position preserved the binding affinity.

Figure 11. Structure of the (S)-(+) lysine salt of phosphonooxymethyl prodrug 24.

Figure 12. Structure, intramolecular H bonding between N-linked C-7 azoles and the 6-AI ring (25–30), and structure of fostemsavir (2).
Recently, a group of researchers tried lattice-based engineering in an effort to identify better conditions for obtaining co-crystal data with HIV-1 entry inhibitors. In this study, small-molecule-based entry inhibitors, including temsavir (26), were examined against the improved lattice (Figure 17).61 From the compounds screened, 40 showed ~100-fold higher potency than 26 against laboratory-adapted HIV-1 strain NL4-3 (EC_{50} values = 0.019 vs 2.2 nM) and ~20-fold higher potency (IC_{50} ≈ 0.002 μM) than that of 26 (IC_{50} ≈ 0.04 μM) against a panel of 30 strains of HIV-1. Interestingly, more than one-half of the virus strains were neutralized with IC_{50} values in the subnanomolar range. Further, 40 showed 10.6-fold higher potency (IC_{50} = 0.0015 μM) than 26 (IC_{50} = 0.0159 μM) against 208 strains of HIV-1. Moreover, in isothermal calorimetry studies, 40 showed 8.6-fold higher affinity toward the HIV-1 env trimer than 26. It was observed that the piperazine ring in 40 adopted a twisted-boat conformation and had higher energy as compared to other derivatives of this class of compounds having a piperidine ring in the chair conformation.
Figure 14. (A) Summary of the structure–activity-guided optimization of HIV-1 entry inhibitors leading to the discovery of fostemsavir. (B) Required structural features of AI-based HIV-1 attachment inhibitors.
Investigation of the cocrystal structures of 40 with the BG505 SOSIP.664 env trimer revealed hydrophilic interactions between the tail of 40 and the BG505 trimer protein. The direct interaction of the N-acylethanolamine tail with the side chains of residues Lys117, Arg429, and Gln432 of the HIV-1 env trimer through its terminal hydroxyl group accounted for the improved potency of 40. In addition, an H-bond was formed between Asp113 of env and the amidic nitrogen on the tail of 40. Furthermore, these functional groups present on the tail can adopt different conformations that are, in turn, useful to accommodate the different conformations of the β20–β21 structural element in different viral clades. Compound 40 was found to be potentially versatile for interacting with HIV-1 env as its tail contains two functional groups that can be either hydrogen-bond donors or acceptors. Thus, investigation of the functional groups on the tail side of other AI analogs may lead to a better understanding of protein-binding interactions.

3.2. HIV-1 Integrase Inhibitors. Integrase strand transfer inhibitors (INSTIs) are a class of antiretroviral agents used to treat HIV-1 infection. Four drugs raltegravir (41a), elvitegravir (41b), dolutegravir (41c), and bictegravir (41d) are used for the treatment of patients preferably in combination with two nucleosides reverse transcriptase inhibitors. HIV-1 integrase (IN) is responsible for catalyzing viral cDNA integration into the host cell genome. Since human cells lack a homologue, HIV-1 IN has been considered an attractive therapeutic target for HIV-1 treatment. However, the appearance of mutations in integrase causes resistance to several IN inhibitors, and the development of better tolerable and more effective drug...
The discovery of improved integrase inhibitors has mainly focused on preventing the incorporation of viral DNA into the host genome by inhibiting the metal-dependent activity (Mg^{2+}/Mn^{2+}) of the integrase-regulated strand transfer (ST) step. The active site of IN adopts an active conformation in which the carboxylate groups of Asp64, Asp116, and Glu152 coordinate two Mg^{2+} ions near the reactive 3′-OH of the viral DNA. One metal ion, coordinated by Asp128 and Glu221, activates the 3′-OH group of the viral DNA for ST. Simultaneously, the other ion, bound by Asp128 and Asp185, destabilizes the scissile phosphodiester group in the target DNA. When used in highly active antiretroviral therapy (HAART), the pyrimidine-based integrase inhibitor raltegravir (41a) demonstrated significant and sustained suppression of viral RNA levels to fewer than 50 copies/mL accompanied by a substantial increase in CD4 immune cell counts. Long-term therapy in HIV-1-infected subjects with 41a induced mutations at amino acids 143, 148, and 155 in integrase together with associated secondary mutations. The Gly155His mutants emerge first and are eventually replaced by Gly148His mutants usually in combination with Gly140Ser. In addition to the above mutations, viral strains isolated from patients also harbor Tyr143Arg and Gln148Arg mutations. These mutations have become the primary cause of resistance to 41a in HIV-1-infected subjects. Elvitegravir (EVG, 41b, Figure 18), which contains a quinoline core and dolutegravir (DTG, 41c, Figure 18), which contains a pyrido[1′,2′:4,5]pyrazino[2,1-b][1,3]-oxazine core, were the next set of drugs approved which have displayed improved efficacy against RAL-resistant strains. However, viral strains that are highly resistant to EVG and DTG demonstrated multiple mutations in the integrase protein. Bictegravir, 41d (2,5-methanopyrido[1′,2′:4,5]pyrazino[2,1-b][1,3]oxazepine) and 41c (Figure 18) share similar functional characteristics and have much higher genetic barriers to resistance. The double mutants Gly140Ser/Gln148His showed higher susceptibility to 41d as compared to 41c.

Earlier literature showed that apart from metal-binding motifs, a hydrophobic aryl chain that coordinates to a proximal hydrophobic pocket via specific interactions is also necessary to inhibit the strand-transfer step. Accordingly, different types of core moieties have been studied, including hydroxylated aromatics, diketo acids, naphthyridine carboxamides, pyrrolloquinolones, dihydroxypyrimidine carboxamides, Al hydroxamic acids, 2-hydroxyisouquinoline-1,3-(2H,4H)-diones, 6,7-dihydroxy-1-oxoisindolines, quinolone-3-carboxylic acids, and carbamoylpypyridines. Al-based carboxylic acids 42a and 42b emerged from further SAR studies, but...
due to their modest activity and good ligand efficiencies (Figure 19A), they were further modified to AI–hydroxamic acids 42c and 42d (Figure 19B).81 These hydroxamic acids have shown a 40-fold increase in potency in enzymatic assays as compared to carboxylic acid-containing compounds and offered structurally simple scaffolds as novel HIV-1 IN inhibitors (Figure 19B).81 However, following metabolic modification that can lead to activation, these compounds can undergo a Lossen rearrangement to yield potentially mutagenic isocyanates. Therefore, to prevent this reaction process, alkylation of either the NH or the OH was explored; however, this resulted in a small loss in potency for 42e and 42f. The N-methylated compound 42e showed stability against oxidative metabolism in human liver micro-

Figure 20. Structure of the 6-AI hydroxamate-based active molecule 44b and its potency.
somes but was rapidly cleared by phase 2 metabolism via glucuronidation in dogs (Figure 19B). Here, increasing the size of the alkyl group did not modify the turnover rate in human hepatocytes and a reduction in antiviral activity was observed. When both N and O-atoms in the AI hydroxamic acid were alkylated, there was a complete loss of HIV-1 integrase inhibitory activity.

Attempts to attenuate glucuronidation rates and improve the metabolic stability, the β position (C-3) of the pyrrolyl ring of the AI nucleus was substituted with amines, ethers, amides, and acyclic C chains.82 The substitution patterns of a of the AI nucleus was substituted with amines, ethers, amides, and acyclic C chains.82 The substitution patterns of a.

The global minimum analysis of simplification of the bicyclic system (Figure 20), conformational eclipsed hydroxamate found to have an allylic-type steric interaction between the piperazin-2-one (Figure 20) was found to be a pyridine N parameters for the respective, in a dog PK experiment. Further, human PK parameters for the N-methyl hydroxamate (42b) were projected from dog PK data, which suggested low blood clearance (Clb = 5.5 mL/min/kg) and moderate bioavailability (F = 41%) and a reasonable half-life (4.5 h). This core showed lower log D values as compared to earlier hydroxamic acid analogs, resulting in an attenuated clearance rate in human hepatocytes.

Since the N-methyl AI hydroxamates 43a and 43b were found to have an allylic-type steric interaction between the eclipsed hydroxamate N-methyl group and the 4-H atom of the pyridine ring of bicyclic systems (Figure 20), conformational analysis of simplified picolinamide-N-methyl hydroxamate was performed in order to evaluate the impact of this interaction on the viral inhibition activity.83 The global minimum calculated for picolinamide-N-methyl hydroxamate (Figure 20) was found to be a pyridine N-H-bonded conformer I (NCCO torsion = −179.42°, ONCO torsion = −178.77°) in which all relevant atoms from the pyridine N to the hydroxamate O were essentially in the same plane. Conformer I was assigned to have ΔE = 0.0 kcal/mol. Among the five possible conformers, the best conformer II, with a constrained coplanar metal-binding domain, showed a very high ΔE value of 5.926 kcal/mol that was higher in energy than I (NCCO torsion = 0.34°, ONCO torsion = 0.32°). Therefore, to overcome this issue, conformational restriction by the introduction of a ring was explored as an approach to restraining the metal-binding motif, resulting in N-hydroxy-dihydronaphthyridinone 44a. This molecule exhibited higher potency and a lower log D value which translated into an improved LipE value. In addition to the six-membered N-hydroxy-dihydronaphthyridinone 44a, the seven-membered ring retained nanomolar biochemical and cellular inhibitory potencies with an IC50 value of 23 nM and EC50 value of 18 nM, respectively. The five-membered ring homologue lost significant potency, presumably due to the modified “bite” angles which would differentially reduce the metal-binding ability (Figure 20). Hence, the six-membered ring was pursued further.83 The above data showed that the locked conformation of the metal-binding motifs contributed significantly to the formation of a stable M(HL)2 complex (where M = Mg2+, Mn2+ and L = ligand) and thus was more beneficial for coordination with the catalytic core of the enzyme.

In the substituted class, the C3-piperidine 44b exhibited a better combination of antiviral potency, membrane permeability, and clearance in both human liver microsomes and human hepatocytes (Figure 20).

Compound 44b showed human PK predictions that were comparable to 43b but offered 300-fold higher antiviral potency, resulting in a significantly lower projected human dose of 32 mg bid that would offer improved safety margins.

Further, substitution of three carbon chains at the C-3 position confirmed tolerance within the pharmacophore but poor bioavailability following oral administration. Therefore, considering each subseries of substitution at C-3, the general trend toward higher LipE values at lower log D was observed, which is of interest and perhaps counterintuitive. The β-substituent is solvent exposed and does not require extensive

Figure 21. (A) Generalized structures with a metal-binding character from the 6-AI series targeted to lower phase 2 metabolism. (B) Dihydronaphthyridinone system containing the active molecule 45e.
In further study, a medicinal chemistry strategy was applied that largely retained the naphthyridinone ring system that efficiently coordinated to bound metal ions (45a) while introducing structural changes that can be effective for improving the extent and rate of conjugation of the N-hydroxyl group (45b and 45d), such as steric hindrance of a hydroxyl group and electronic deactivation or removal of this group (45e, Figure 21A). On the basis of these points, substitutions around the dihydronaphthyridinone and benzyl systems were made.

In this series, 45e (Figure 21B) was designated a potent ST inhibitory candidate (IC₅₀ = 40.5 nM) that showed no activity against DNA polymerase α/β, targeted human liver microsome stability, and appreciable aqueous solubility. Compound 45e successfully inhibited laboratory strains and patient viral isolates cultured in peripheral blood mononuclear cells.

No significant inhibition of ion channels, receptors, enzymes, or transporters in a CEREP broad ligand-screening panel was observed with 45e. In addition, an advantage in terms of potency over the approved ST inhibitors 41a and 41b was found compared with that of the wild type for all of the mutants tested. Compound 45e (Figure 21B) exhibited a <10-fold difference in potency compared with that of the wild type for all of the mutants tested with one exception, the double-mutant Gly140Ser/Gln148Lys virus. However, due to its projected short half-life, 45e was not further pursued for clinical development.

On the basis of the studies explained above, we propose a 6-Al-containing planar heteroaromatic two-metal-binding pharmacophore model to design potent integrase inhibitors (Figure 22). The additional nitrogen atom of the 6 Al nucleus interacts with the Mg₂⁺ or Mn²⁺ ions through coordinate bond formation present at the active site of IN enzyme. The nitrogen and oxygen atoms are known to be hard Lewis bases, while Mg²⁺ is a hard Lewis acid. The hard Lewis bases act as donors, and the acids act as acceptors. At the N-1 position, a flexible linker of 2–3 carbon atoms and an aromatic group that extends into an adjacent hydrophobic space are crucial for designing a potent inhibitor chemotype.

**HUMAN ORTHOPNEUMOVIRUS/RESPIRATORY SYNCYTIAL VIRUS (RSV)**

Respiratory syncytial virus (RSV) is a respiratory pathogen that belongs to the Paramyxoviridae family with a single-stranded, negative-sense RNA (15.2 kb) genome. RSV is the main cause of bronchiolitis and acute lower respiratory tract infection (ALRTI) in infants, adults, and immunocompromised patients. The viral envelope is comprised of three proteins: the fusion protein (F), attachment glycoprotein (G), and small hydrophobic (SH) protein. The virulence of RSV occurs mainly through the surface proteins F and G. The initial step of RSV attachment to the host is carried out by the G protein, while the F protein mediates RSV envelope fusion, which releases the viral genome into the host cell, while the role of the SH protein in either process is somewhat enigmatic.

There are two subtype strains, RSV-A (639 bp) and RSV-B (724–762 bp), which are defined by the nucleotide sequences in the ectodomain of the F protein. As the RSV F protein is a key player in viral infection, it is imperative to discuss the structure and mechanism of this protein.

**RSV Fusion Protein.** The RSV F protein is a glycoprotein that after proteolysis is comprised of two subunits, a 55 kDa, carboxy-terminal F₁ subunit (137–574 residues) and a 15 kDa amino-terminal F₂ subunit (26–109 residues). The F₁ and F₂ elements are covalently connected via disulfide bonds to form a heterodimeric protomer. Three F₁/F₂ heterodimers associate...
converts into postfusion conformation. The unstable prefusion conformation of RSV F is refolded into the postfusion conformation. This process of refolding is irreversible, and temperatures increase the conversion to the postfusion conformation at >90 °C.

Membrane fusion.

Figure 23. Simplified 2D ligand interaction of JNJ 53718678 (46b) with prefusion RSV F. RSV F is shown as a molecular surface with three identical protomers, each shown in a different color (Fα, green; Fβ, pink; Fγ, tan). JNJ 53718678 is shown as a ball-and-stick representation with carbon atoms colored in gray, nitrogen atoms in blue, oxygen atoms in red, chlorine atoms in dark green, fluorine atoms in light blue, and sulfur atoms in orange. (Inset) Magnified view of the binding of JNJ 53718678 into the central cavity. Conversion of 46b into different benzimidazoles (47, 48, and 52) and AI isomers accommodating respiratory syncytial virus (RSV) fusion inhibitors (49–51).

to form the mature trimeric form of the F protein. The trimerization results in a spheroidal-shaped prefusion conformation of the F protein heterodimer. The unstable prefusion conformation of F is refolded into the postfusion conformation. During the refolding, the fusion peptides are withdrawn from the central cavity and projected away from the viral membrane. If another membrane, such as a host cell membrane, is in close proximity, the fusion peptides will insert into the membrane with the F protein thus binding to both membranes. The prefusion confirmation is highly unstable and proceeds to refold via association of the heptad repeats present in the amino and carboxyl termini of the F subunit. Once again, a trimer of hairpins is created that helps to pull the viral membrane and host membrane together to promote membrane fusion.

The postfusion confirmation of F is highly stable and melts at >90 °C. From the literature, it is well known that at some basal rate the unstable prefusion confirmation of RSV F converts into postfusion confirmation. It was observed that both longer incubation times and incubation at elevated temperatures increase the conversion to the postfusion confirmation.91,92 This process of refolding is irreversible, giving a stable postfusion confirmation of F protein.

There have been a number of mechanisms proposed for the entry of RSV in host cells. RSV-infected cells fuse with neighboring cell membranes to generate multinucleated cells called syncytia. A more recent report indicated that the initial steps of RSV fusion occur at cholesterol-rich microdomains in the plasma membrane.93 A subsequent study demonstrated that RSV utilizes macropinocytosis as an initial entry mechanism followed by fusion in endosomes.94 Thus, the evidence now suggests either a two-step fusion event or fusion in endosomes after macropinocytosis. However, it may be the case that RSV can fuse at both the plasma membrane and in endocytic vesicles with different efficiencies depending on the environmental conditions and target cells. Other options for the fusion mechanism, i.e., provocation by a second viral glycoprotein94,95 and a clamp model,96 also were not reassuring.

The current belief is that the infectious cycle RSV starts with virion attachment to the apical surface of polarized, ciliated airway epithelial host cells. Subsequently, the viral fusion (F) glycoprotein causes fusion of the viral and host cell membranes by undergoing a drastic conformational change. After fusion, the helical ribonucleoprotein complex (RNP) is released into the host cell cytoplasm followed by replication and transcription which occur in the cytoplasm in viral inclusion bodies that produce viral products. The viral RNA-dependent RNA polymerase (RdRp) complex is responsible for transcribing viral mRNA and synthesizing positive-sense nongenome intermediates required for replication of new negative-sense genomes for packaging into virions.

Clinical Intervention To Inhibit RSV Infection. There has been a consistent attempt to develop an effective treatment regimen to control RSV infection since the discovery of the virus in the 1950s. Palivizumab (Synagis) is a humanized IgG-1 mAb that binds the RSV-F protein A epitope and is administered as an intramuscular injection.86,98 IgG antibodies, which are involved in the secondary immune response, have a half-life of approximately 20 days. Palivizumab offers greater activity against RSV and the relative ease of administration of a smaller volume of drug as an injection, compared with RSV-IGIV (RSV immune globulin intravenous, a polyclonal IgG product with a high content of anti-RSV IgG). This drug was approved for the treatment of infants aged less than 2 years who are suffering from RSV with hemodynamically significant congenital heart disease (HSCHD). It provides immunoprophylaxis against serious lower respiratory tract infections (LRTIs) caused by respiratory syncytial virus (RSV).96 Ribavirin (46a), discovered in 1972 by Witkowski and co-
workers, is a guanosine analog that exhibits broad-spectrum activity against several RNA and DNA viruses (Figure 23). Although originally approved only for the treatment of severe RSV infection in children, it was later used for the treatment of other viruses. Ribavirin (46a) suffers from low efficacy and requires an aerosol/intravenous (IV) mode of administration. It is also teratogenic so poses a threat to healthcare workers who may be exposed to ribavirin aerosols.

On the basis of phenotypic screening of chemical libraries, several novel small-molecule-based RSV fusion inhibitors have been identified. Subsequently, on the basis of crystallographic investigations, it was observed that these small molecules bound to a fusion intermediate of F and prevented the formation of the postfusion conformation. To date, all known RSV fusion inhibitors bind in the same pocket and have the same mechanism of action. In addition to stacking interactions, other interactions between the protein and 46b were observed. The 5-Cl group of 46b interacts with the carbonyl oxygen of Thr397 through a halogen bond along with a water-mediated H-bonding interaction between the O atoms of the sulfone and the side chain of Arg339. In addition to the above, it has been observed that rearrangement of the side chains of Phe140, Phe488, and Phe137 is required to make a hydrophobic environment to trap the CF3 group of 46b.

The 5-chloroindole heterocycle is involved in π–π stacking interactions with Phe488A, Phe488B, and Phe140B and in a weak C–H/π interaction with Phe140B, which are present between RSV F protomers A and B (FA and FB, respectively) of lobe 1. Similarly, the 1,3-dihydroimidazo[4,5-c]pyridin-2-yl group also forms a π–π stacking interaction with Phe488A and Phe488C and a weak C–H/π interaction with Phe140A, which are present between RSV F protomers A and C (FA and FC, respectively) of lobe 2 (Figure 23). The formation of these aromatic protein–ligand stacking interactions seems to be a commonality between all known RSV fusion inhibitors and may lock the central heterocyclic moieties of these inhibitors in a fixed conformation. In addition to stacking interactions, other interactions between the protein and 46b were observed. The 5-Cl group of 46b interacts with the carbonyl oxygen of Thr397 through a halogen bond along with a water-mediated H-bonding interaction between the O atoms of the sulfone and the side chain of Arg339. In addition to the above, it has been observed that rearrangement of the side chains of Phe140, Phe488, and Phe137 is required to make a hydrophobic environment to trap the CF3 group of 46b.

The C3 substitution in the benzimidazolone with a CF3 group in 46b, instead of the cyclopropyl moiety in 47, contributed to improved metabolic stability and decreased susceptibility to reactive metabolite formation. Compound 46b exhibited a mean EC50 = 0.46 nM against recombinant rgRSV224 virus in HeLa cells, whereas it showed an EC50 ≈ 0.2–20 nM against 8...
nonrecombinant RSV-A and RSV-B strains in plaque reduction assays.86

Structural comparison of JNJ 53718678 (46b), which possesses an indole moiety, with BMS-433771 (47) and JNJ-49153390 (48), both of which have a benzimidazole core (Figure 23), implied that the central heterocyclic core could be replaced without any loss of antiviral activity provided that the heterocycle offered an opportunity for π-stacking.102 Encouraged by this finding, 4-, 5-, and 6-azaindole cores were explored as part of the optimization process (Figure 23). While offering similar antiviral potency (∼ EC50 = 1 nM), the 5-chloro azabenzimidazole compounds 49 and 50 displayed very different PK profiles, especially with regard to their distribution to the lungs. However, installing a nitrogen atom at C5 in the indole renders 51 more basic, which alters the pKa and lipophilicity, resulting in improved pulmonary permeability and distribution.104 According to a reported tissue distribution model, basic amines with a pKa value of >8 show better uptake,105 which may be the reason for increased uptake of 51.

Compound 51, a 5-azaindole analog of 46b, showed high binding affinity to the RSV F protein, indicating that the introduction of a nitrogen atom at C-5 may compensate for halogen bonding. The azaindole moiety exhibited improved π-stacking with Phe488/Phe140.102 Compound 52 was synthesized having 3-(methylsulfonyl)-6-azaindole as a C-2 substituent instead of the benzimidazole in 46b (Figure 23) and found to have 8–10-fold lower potency (EC50 = 78 nM) when compared to 46b.106

Furthermore, a survey of pharmacophores of RSV inhibitors indicated that indole 53a, azabenzimidazole 53b, and 5-azaindole 53c exhibited almost comparable EC50 values in the RSV cell-based assay107 similar metabolic stability in human liver microsomes (Figure 24A), and similar membrane permeability in human cell lines. A significant difference in their plasma protein-binding profiles was observed reflecting their differential lipophilicity. The indole analog 53a exhibited high protein binding in human plasma (3.6% free fraction), whereas azabenzimidazole (53b) and 5-azaindole (53c) were distinctly less protein bound (30% and 47.2% free fractions, respectively). The reduced plasma protein binding of azabenzimidazole 53b and AI 53c could be attributed to the combined effect of the lipophilic modulation caused by trifluorination at the terminal carbon of the N-1 propyl chain and the newly installed nitrogen atoms in the core ring compared with indole 53a.107 In addition, 53c was well distributed to the lungs since it has a basic character. Another synthetic analog, 53d, showed an EC50 value in the nanomolar range in an RSV A2 assay hosted by HEP-2 cells (Figure 24B). Subsequently, the X-ray co-crystallographic structure of 53d in complex with RSV-A2 suggested that the presence of allylic strain induced by the amide bond forces the C-2 heteroaryl substituent on the piperidine ring, i.e., pyrazolopyrimidine, to formation of a dihedral angle of 95°.108

On the basis of the above results, we propose a pharmacophore model having a central fused heterocyclic core system that engages in aromatic stacking interactions with the prefusion conformation of the RSV F protein as necessary for optimum inhibitory activity against the virus (Figure 24C). In addition, a heteroaromatic ring at the C-2 position of the core system that imparts conformational flexibility should be attached through a linker (Figure 24C).108
Figure 26. Scaffold hopping from an indole-derived inhibitor (54e) to a 6-azaindole core (55a) and benzimidazole core (55b) as neurotropic alphavirus replication inhibitors. MDR1 recognition was assessed by measuring rhodamine uptake in the presence of the MDR inhibitor tariquidar (5 μM) and either 30 μM of the test compound or vehicle and calculating (C_{av} - C_{veh}) × 100/(C_{av} - C_{veh}), where C_{av} is the concentration of rhodamine 123 in the presence of an antiviral inhibitor, C_{veh} is the concentration in the presence of a vehicle, and C_{veh} is the concentration of rhodamine 123 in the presence of tariquidar.

NEUROTROPIC ALPHAVIRUSES

Neurotropic alphaviruses or encephalitis viruses, including Western equine encephalitis virus (WEEV), are transmitted by mosquitoes and infect neurons present in animals and the human CNS.109 The positive-stranded m-RNA (12 kb) alphavirus is enclosed in an isocahedral nucleocapsid that is surrounded by a lipid envelope coated by a viral envelope containing two glycoproteins, E1 and E2. These two proteins form a stable heterodimer, and three E2−E1 heterodimers interact to form the spike required for viral infection. The alphavirus normally enters through endocytosis in clathrin-coated vesicles followed by transfer to endosomes, where the low pH results in a conformational change in the E1−E2 heterodimer such that the fusion domain in E1 is exposed and the virus envelope fuses with the endosomal membrane.109

In the alphavirus class, Semliki Forest virus (SFV) and Sindbis virus (SIN) are widely used as models to study different steps of pathogenicity, including virus entry, endosomal release, and budding inside the host cells.110 According to recent studies; neurotropic alphaviruses can also propagate without capsids. Neurotropic alphaviruses are considered category B priority pathogens by the National Institute of Allergy and Infectious Diseases (NIAID) because they can be aerosolized and released into a population center as potential bioterrorism agents, causing CNS infections that lead to potentially fatal encephalitis. Another study showed that WEEV activates transcription factor, interferon regulatory factor 3 (IRF-3) mediated neuronal innate immune pathways, and any revocation in IRF-3 will cause enhanced virus-mediated injury.111 Compounds carrying thienopyrrole (54a−d),112 indole-2-carboxamides (54e and 54f),113,114 and pyrrole-2-carboxamide (54g)115 have been reported to act as alphavirus replication inhibitors (Figure 25). The thieno[3,2-b]pyrroles were identified as a class of potent antiviral agents via a high-throughput screening (HTS) campaign. The replicon-based assay used for the HTS and subsequent validation steps implicated viral replicase proteins as potential targets of these thieno−pyrroles. In the study, the analogues were evaluated with the cell-based WEEV replicon assay in which the majority of the WEEV structural genes are replaced with the firefly luciferase gene as a reporter for viral RNA replication. An MTT assay was performed to evaluate their effects on cell viability (Figure 25). This study produced 54a which exhibited an IC_{50} of 24.4 μM and a CC_{50} of >100 μM. Further, structural optimization of 54a started from substitution of the equipotent benzyl amide instead of the 2-furanylmethyl amide to obtain 54b.113 The 4-fluorobenzyl group of 54b was changed to a 4-chlorobenzyl group to afford 54c. The chiral molecule 54d was synthesized using (R)-α-methylbenzylamine and inhibited the virus with an IC_{50} of 8.3 μM. Replacement of the thieno[3,2-b]pyrrole core with an indole core yielded 54e. Compound 54e demonstrated moderate potency, stability toward oxidative metabolism, and protective effects that correlate with both in vitro and in vivo antiviral activity. The indole analog 54e with chirality at the benzylamine expressed the same eudismic ratio difference in pharmacological activity between the two enantiomers as observed with the thieno[3,2-b]pyrroles with the (R)-enantiomer having a superior IC_{50} value compared to the (S)-enantiomer. Interestingly, 54f and 54g showed a 10-fold improvement in IC_{50} values, suggesting that an ethylene linker along with the pyridine ring plays a major role in the inhibitory activity of these molecules.114 Furthermore, upon moving the nitrogen of the pyridine ring from the para to the ortho position, a 40-fold decrease in potency was observed. The pyrrole in 54g is a viable substitute for the indole core of 54f and reduced the molecular weight and actually diminished the cytotoxicity compared with that of 54f.

Subsequently, biosystemic replacement of the indole core in 54e with an AI to provide 55a was examined. The 6-azaindole derivative 55a exhibited reduced lipophilicity and enhanced solubility while retaining antiviral potency (Figure 26).114 The major efflux transporter at the blood−brain barrier (BBB), P-glycoprotein (P-gp/MDR1), facilitates xenobiotic efflux from the CNS. The degree to which P-gp interacts with the molecules was measured using a rhodamine 123 uptake assay conducted in MDCK cells transfected with human P-gp (MDRI-MDCKII). Rhodamine-123 is a known P-gp substrate that is actively effluxed from MDRI-MDCKII cells. Significantly, the 6-azaindole-derived 55a was found to be more effective in attenuating P-glycoprotein (P-gp/MDR1) recognition. The MDR recognition value was reduced to 1, suggesting minimal efflux of 55a from cells.

Compound 55a exhibited moderate antiviral activity, IC_{50} = 4.4 μM, and was not able to effectively reduce the viral load (69.4 pfu/mL) as compared with 54e (39.3 pfu/mL).114 Moreover, an eroded metabolic stability and increase in solubility was observed upon replacement of the indole ring of 54f with the pyrrole in 54g, suggesting that the central aromatic ring is a major site of metabolism. In addition, the improved metabolic stability shown by replacement of the
indole of \textit{54f} with the more electron-deficient benzimidazole ring in \textit{55b} is also consistent with this proposal. A 5-fold decrease in potency was noted when a propyl linker was used with a pyridine ring instead of an ethylene linker of \textit{54f}.\textsuperscript{114} This suggests that the optimum distance between the amide group and the pyridine ring may be a significant structural requirement for potent antiviral molecules.

A complete loss of antiviral activity was observed upon removal of the aromatic core from any molecule, which indicates the necessity of an aromatic core. The \(p\)-halogenated benzyl ring at the N-1 position of the aromatic core and a linker connecting it to the aromatic core are important to achieve significant inhibitory potency. Considering all of the above findings, we propose the general concept for a pharmacophore model to develop a potent neurotropic alphavirus inhibitor depicted in Figure 27.

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**Figure 27.** Proposed pharmacophore model for neurotropic alphavirus inhibitor.

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### DENGUE AND EBOLA VIRUSES

Dengue virus (DENV) belongs to the \textit{Flaviviridae} family and is classified as an ‘arbovirus’ since it is transmitted by arthropod vectors, particularly mosquitoes, such as \textit{Aedes aegypti} and to a lesser extent \textit{Aedes albopictus}.

The genome of DENV is a single-stranded, positive-sense RNA. There are four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, and each interacts distinctly with antibodies in human blood serum.\textsuperscript{117} Currently, no specific antiviral drugs are available to treat DENV. A live-attenuated tetravalent vaccine, Dengvaxia (CYD-TDV), has been developed by Sanofi-Aventis Pasteur Limited, Paris, France, and approved in endemic countries; however, it exhibits suboptimal protection against DENV-1 and DENV-2.\textsuperscript{118} The vaccine’s varying efficacy across different ages and serostatus as well as a clear safety signal in seronegative recipients, i.e., that Dengvaxia enhanced subsequent disease in some seronegative individuals, raised a number of questions against other dengue vaccine candidates also. Therefore, new therapeutics against DENV are needed.

Ebola virus (EVD) belongs to the \textit{Filoviridae} family and has a single-stranded, negative-sense RNA genome. To date, no specific antiviral medications or approved vaccines are available for EVD.\textsuperscript{116}

**Adaptor-Associated Kinase 1 (AAK1 Inhibitors).**

Originally, AAK1 inhibitors were developed to treat neurological disorders and only later emerged as antiviral agents. Verdonck and co-workers developed AAK1 inhibitors with broad-spectrum antiviral properties.\textsuperscript{119} Their work is based on targeting the host kinases used by viruses for intracellular membrane trafficking aiding their entry into host cells. Intracellular trafficking of many RNA viruses is regulated by clathrin-associated host adaptor proteins controlled by AAK1 and cyclin G-associated kinase (GAK). Both are serine-threonine kinases belonging to the NUMB-associated kinase (NAK) family. Anticancer drugs such as sunitinib and erlotinib inhibit AAK1 and exhibit broad-spectrum in vitro antiviral activity against different viruses, including HCV, DENV, Zika virus, and West Nile virus.

Compound \textit{56}, a 7-AI derivative (Figure 28), was used as the basis to develop a series of potent AAK1 inhibitors.

Compound \textit{56} was 3-fold more selective for AAK1 than GAK and 8- and 22-fold more selective for AAK1 than bone threonine kinases belonging to the NUMB-associated kinase (NAK) family. Anticancer drugs such as sunitinib and erlotinib inhibit AAK1 and exhibit broad-spectrum in vitro antiviral activity against different viruses, including HCV, DENV, Zika virus, and West Nile virus.

Chapter 28. Structures of known adaptor-associated kinase 1 inhibitors \textit{56} and \textit{61}, broad-spectrum kinase inhibitor \textit{K252a} (\textit{62}), and tyrosine kinase inhibitor nintedanib \textit{63}. Terminal cyano group and C3 position of \textit{56} were optimized to afford 7-azaindole analogs \textit{57} and \textit{58}. Replacement of C-2 and C-4 with a N atom in analogs \textit{59} and \textit{60}.
morphogenetic protein 2 inducible kinase-2 (BMP2K) and serine/threonine-protein kinase 16 (STK16), respectively. Furthermore, the binding mode of S6 to AAK1 based on X-ray studies showed that its 7-AI ring directly bound between the side chain of Ala72 from β2 on the kinase N-lobe and Leu183 of the C-lobe. N-1 and N-7 of S6 engaged in H-bonding interactions with Asp127 and Cys129 at the kinase hinge region. Similarly, the terminal aromatic nitrogen of the pyridine moiety formed a H bond with the side chain of Lys74; however, the nitrogen atom of the terminal cyano group interacted with the side chain of Asn136 (Figure 29).

Figure 29. Binding interactions of compound S6 in the ATP-binding pocket of AAK1. Terminal cyano group, pyridine nitrogen, N-1, and N-7 of AI are involved in hydrogen-bonding interactions with amino acid residues Asn136, Lys74, Asp127, and Cys129, respectively, around the ATP-binding pocket.

The SAR studies with S6 first explored the effect of replacing the 5-(4-cyanophenyl) group with a phenyl, thienyl, and substituted phenyl ring carrying electron-donating and -withdrawing groups and a halogen. Compounds carrying a 3,4-dimethoxyphenyl ring at the C-5 position (S8) showed stronger AAK1 affinity (IC50 = 0.00432 μM) than the positive control sunitinib (IC50 = 0.0474 μM) (Figure 28) and exhibited good activity against DENV-2 (EC50 = 1.64 μM). Further focus was given to replacing the N-acyl moiety of S8 with aromatic, heteroaromatic, and cycloalkyl groups. It was observed that the 3-pyridyl moiety S8 was critical for AAK1 binding as other N-acyl analogs showed a 100-fold decrease in AAK1 affinity compared with S8. However, the amide bond of the N-acyl group in S8 could be replaced with aryl keto, phenyl, and alkynyl groups without a significant loss in AAK1 affinity. The 3-ethynylpyridine analog S7 was found to exhibit excellent AAK1 binding (IC50 = 0.00402 μM) and antiviral potency against DENV-2 (EC50 = 0.72 μM). Scaffold modification of 7-AI (S7) to pyrrolo[2,3-b]pyrazine (S60) showed potent AAK1 binding with an IC50 = 0.00927 μM; however, it also demonstrated greater cytotoxicity. In parallel, scaffold modification of the 7-AI S8 to the pyrazolo[3,4-b]pyridine S59 resulted in a 100-fold reduction in AAK1 binding affinity (IC50 = 0.462 μM) compared with S8. This study suggested that S7 (AAK1 IC50 = 0.00402 μM; DENV-2 EC50 = 0.72 μM; EBOV EC50 = 1.59 μM) and S8 (AAK1 IC50 = 0.00432 μM; DENV EC50 = 1.64 μM; EBOV EC50 = 4.24 μM) were the optimized analogs of S6. However, S6 did not show any significant inhibitory activity toward EBOV (EC50 > 10 μM). Furthermore, S6 and S7 were advanced to studies in human primary dendritic cells, which are physiologically more relevant models for DENV infection. Compounds S7 and S8 showed dose-dependent inhibition of
Figure 31. Representative examples of reported AAK1 inhibitors.

Figure 32. (A) Modification of the acylaminothiazole moiety with heterocycles carrying a common H-bonding motif in the bicyclic core (except in compound 73). (B) Two-dimensional interaction diagram of PI3Kγ inhibitor 69.
DENV-2 infection with EC_{50} values of 0.0428 and 0.739 μM, respectively. Finally, in the kinase selectivity experiment against 468 kinases, 57 displayed binding with multiple kinases, including NAK family members, which may explain the broad-spectrum antiviral effect of 57. Compound 57 showed lower cytotoxicity (CC_{50} > 20 μM) toward Huh7 mammalian cells in \textit{in vitro} in cell viability assays. Overall, this work demonstrated that the development of cellular AAK1 inhibitors may represent a promising broad-spectrum antiviral strategy.

7-AI is a well-established hinge-binding scaffold that can adopt different orientations in the folding cleft of the hinge region of the kinase.^{120} The capacity of 7-AI to engage in H bonding and its amide isosteric characteristics can be tactically applied in drug discovery studies. For example, the binding interaction of the broad-spectrum kinase inhibitor K252a (62), the tyrosine kinase inhibitor nintedanib (63), and 56 with the AAK1 binding pocket demonstrated the versatility of AIs (Figures 28 and 30).^{121} Despite the differences in their chemical structures, the pyrrole-2-one moieties of AKA1 inhibitors share different functional groups at the correct position may bind similarly to a protein.

A few AAK1 inhibitors that are non-7-azaindole chemotypes carrying pyrazolopyrimidine (64), pyrrolopyrimidine (65 and 66), and imidazolopyridazine (67 and 68) pharmacophores are shown in Figure 31. These isomeric pharmacophores incorporate three nitrogen atoms that are positioned differently, and these nitrogens may be (no X-ray data available) interacting with different amino acid residues at the ATP-binding pocket depending on their substituents to afford a signatory H-bonding pattern of AAK1 inhibitors. A few phosphoinositide-3-kinase γ (PI3Kγ) inhibitors share common H-bonding donor and acceptor motifs (Figure 32).^{122} The bicyclic ring in inhibitors engages in a bidentate manner with Val882, a hinge motif of PI3Kγ. Hence, this bidentate interaction is a determining factor of the potency of different substituted isoindolenones (Figure 32).^{123,124} Replacement of the acylaminothiazole ring of 69 with a 7-AI yielded 71 and 74. Both of these compounds exhibited excellent inhibitory potency with IC_{50} values of 0.05 and 3.3 nM, respectively, as compared to the naphthyridinone 70, the azaindole-2-one 72, and the pyrazolopyridines 73 and 75. The NH in the azaindazole 73 is not suitably oriented for interaction with Val882, and this molecule displayed 5.4-fold lower potency (IC_{50} = 0.49 μM) than the isonitrogue 75 (IC_{50} = 0.09 μM). Subsequently, the 7-AI analog 71 was found to exhibit greater potency (IC_{50} = 0.05 μM) than reference compound 69. Substituting a benzyl acetic acid derivative at the C-3 position in 71 provided very potent PI3Kγ inhibition (IC_{50} = 3.3 nM), and this molecule exhibited an acceptable pharmacokinetic profile in rats (in vivo CL = 1.8 L/h/kg). Thus, the combination of C-3 substitution and the versatility of the N1 and N7 nitrogen in forming bidentate interactions with the target site allows for facile optimization.

Currently, AAK1 inhibitors are envisioned to be beneficial in countering SARS-CoV-2 entry into host cells. Compounds 56–58 and their derivatives can be further optimized and tested against various virus families, including SARS-CoV-2. Repurposing kinase inhibitors as broad-spectrum antiviral agents is a recent and ongoing endeavor.^{115–128} It has been suggested that kinase inhibition may significantly surpass viral resistance because the virus does not genetically control the host kinases. However, toxicity is a major concern associated with kinase inhibition since host kinases play essential roles in mediating other cellular functions.^{119}

### HEPATITIS C VIRUSES

Hepatitis C virus (HCV) is a small enveloped RNA virus composed of 9.6 kb RNA having a long open reading frame (ORF) flanked by a 5′- and 3′-nontranslated region. It belongs to the \textit{Flaviviridae} family, and its genome consists of single-stranded, positive-sense RNA.\textsuperscript{129} The 3′-nontranslated region contains a poly U/UC tract and a highly conserved 98-nucleotide element that is required for viral RNA synthesis. The 5′-nontranslated region is the most conserved among the different genotypes and contains the internal ribosomal entry site (IRES) element which is essential for a direct Cap-independent translation of the ORF region. Translation results in a precursor polyprotein containing ~3000 amino acids (aa). The precursor polyprotein gets processed by the host cell and viral proteases into both structural (S) and nonstructural (NS) proteins, respectively. The S proteins are comprised of a nucleocapsid core (C) and the glycosylated transmembrane proteins E1 and E2 required for the attachment of HCV to host cell receptors and the viroporin p7 that likely forms ion channels essential for assembly and release of infectious virions. The other precursor protein contains the NS proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The nonstructural proteins play a major role in replication of the HCV virus. Out of all of the above-mentioned nonstructural proteins, NS4B represents a druggable and appealing antiviral target, thus making NS4B the last entry target in the HCV drug discovery process. Although a number of direct antivirus agents were approved against HCV, none of the drugs have been approved against NS4B to date.\textsuperscript{130,131}

### HCV NS4B Inhibitors

Chen and co-workers developed 7-azaindole-based compounds that target the nonstructural, membrane-bound protein NS4B, an integral hydrophobic membrane protein that plays a pivotal but undefined role in the HCV RNA replication mechanism.\textsuperscript{132} All of these compounds were evaluated for activity against the genotype 1b HCV replicon by measuring mRNA levels with respect to cellular GAPDH mRNA in Huh-7 cells. Briefly, quantitative RT-PCR was performed to quantify the amount of intracellular HCV RNA, and the concentration of a compound inhibiting HCV RNA replication by 50% (EC_{50}) is indicated. The indolopyridine-sulfonamide 76, which exhibited an EC_{50} of 7 nM,\textsuperscript{133} was chosen as the lead compound, and a SAR study was performed by replacing the indole ring with isomeric 4-, 5-, 6-, and 7-azaindoles to explore anti-HCV activity (Table 4).\textsuperscript{132}

The SAR exploration revealed that the 7-azaindole series with an electronegative CF_{3} substituent at C-5 (77) exhibited more potent anti-HCV activity than the indole analog 76.\textsuperscript{132} Analogs 78 and 81 carrying small lipophilic substituents on the azaindole ring were also found to exhibit good HCV inhibition. However, the unsubstituted 7-azaindole analog 80 was less active (Table 4). Similarly, attaching propoxy (85) and isopropoxy (86) substituents at the C-5 position on the 7-azaindole core was found to be inferior for anti-HCV activity. Shifting the nitrogen around the aromatic ring to form isomeric As 82–84 with a small lipophilic methyl group at either C-5 (82) or C-6 (83, 84) resulted in a significant drop in potency. In pharmacokinetic screening, oral administration...
of a single 10 mpk dose of the 5-CF₃ analog 77 to rats showed excellent exposure (AUC₀−₂₄h = 5833 nM·h) of the compound. It also exhibited higher exposure (AUC₀−₂₄h = 8162 and 5440 nM·h, respectively) in dogs (PO, 2 mpk) and monkeys (PO, 3 mpk) than 76 (AUC₀−₂₄h = 6858 and 7568 nM·h, respectively). Notably, the C-2 position of AI has been reported to be vulnerable to oxidative metabolism, leading to C-2 hydroxy metabolites. However, the C-2 position is occupied in optimized compound 77, thus, C-2 oxidative metabolism may be avoided.

Table 4. Structure of Indole Analog 76 (Selective Inhibitor of HCV RNA replication) and Its AI Analogs 77−86

| Compd No | Structure | EC₅₀ (gt 1b) | HCV subgenomic RNA replication assay | Ring type |
|----------|-----------|--------------|-------------------------------------|-----------|
| 76       | ![Structure Image] | EC₅₀ = 7 nM | Indole                              |           |
| 77       | ![Structure Image] | EC₅₀ = 2 nM | 7-azaindole                         |           |
| 78       | ![Structure Image] | EC₅₀ = 13 nM | 7-azaindole                         |           |
| 79       | ![Structure Image] | EC₅₀ = 87 nM | 7-azaindole                         |           |
| 80       | ![Structure Image] | EC₅₀ = 217 nM | 7-azaindole                         |           |
| 81       | ![Structure Image] | EC₅₀ = 15 nM | 7-azaindole                         |           |
| 82       | ![Structure Image] | EC₅₀ = 1400 nM | 6-azaindole                        |           |
| 83       | ![Structure Image] | EC₅₀ = 230 nM | 5-azaindole                         |           |
| 84       | ![Structure Image] | EC₅₀ = 119 nM | 4-azaindole                         |           |
| 85       | ![Structure Image] | EC₅₀ = 2150 nM | 7-azaindole                        |           |
| 86       | ![Structure Image] | EC₅₀ = 7600 nM | 7-azaindole                        |           |

*EC₅₀ values are the averages of at least two independent determinations. HuH7 cells harboring genotype 1b (gt 1b) HCV bicistronic replicons were plated at 5000 cells/well in 96-well plates. Compounds were added to the wells with a final DMSO concentration of 0.5%.*

Reports of different chemical classes targeting HCV NS4B, including benzimidazole 87, pyrazolopyrimidine 88, pyrazolopyridines 89 and 90, and imidazothiazole 91 have emerged (Figure 33). The suggestion of a common ligand-binding site on NS4B is unclear due to the lack of crystal structure data on the NS4B protein, which hinders structure-based drug discovery.

I. INFLUENZA A VIRUSES

Influenza virus belongs to the Orthomyxoviridae family of viruses, which have a negative-sense, single-stranded, and segmented RNA genome with diverse antigenic characteristics. Current subtypes of influenza A viruses that routinely circulate in humans include A(H1N1) and A(H3N2) types. Although vaccination represents the best way to lessen the impact of the disease, the virus varies continuously due to antigenic drift that can evade pre-existing immunity. Thus, influenza vaccines are reformulated every year to match circulating strains. Currently, oseltamivir 92 (oral), zanamivir 93 (inhalation), peramivir 94, and baloxavir-marboxil 95 (oral) are FDA-approved antiviral drugs that are recommended for the treatment of influenza virus infection (Figure 34). At the same time, zanamivir and oseltamivir are also recommended for chemoprophylaxis.

Generally, anti-influenza drugs work best only when they are administered in a timely manner within 48 h of the onset of infection. The current antiviral standard of care (SOC) for the treatment of influenza is the neuraminidase inhibitors, oseltamivir, 92 (Figure 34), and zanamivir, 93, 136 Zanamivir, 93, has low oral bioavailability; hence, it is given via the topical route by inhaler, but 92 is administered orally. These drugs are effective against a variety of type A and B influenza viruses, but there are three major limitations to these molecules that have emerged in recent years. First, the neuraminidase inhibitors have only a moderate impact on the severity of symptoms as well as the duration of illness, and they must be administered within 24−48 h of the onset of infection. Second, infants suffering with influenza in a few countries have recently shown the emergence of viruses with mutations in the neuraminidase gene that encode for drug-resistant neuraminidase proteins. If this frequent emergence of resistant mutants is found to be a general occurrence in children, it represents a serious concern, especially since children are an important source of the spread of influenza in the community. Third, recent reports about H5N1 influenza virus have shown resistance to oseltamivir. Hence, new anti-influenza therapeutics with a novel mechanism of action are required.

Novel Polymerase Basic Protein 2 (PB2) Inhibitors of Influenza A Virus. The discovery of alternative therapeutic options for the treatment of influenza virus are still a challenge, and phenotypic-assay-based drug discovery efforts have been made by a number of research groups in an effort to identify novel chemotypes. The viral polymerase is made up of three different subunits, i.e., PB1, PB2, and PA. The heterotrimeric viral polymerase synthesizes viral mRNAs via a cap-snatching mechanism where it utilizes host pre-mRNA as a primer for transcription. The PB2 subunit contains a cap-binding domain that recognizes 7-methyl GTP (m7 GTP) on the 5′-end of the host pre-mRNA. Once host pre-mRNA is bound to PB2, the PA endonuclease subunit cuts the host RNA strand, leaving behind a 10−13-nucleotide primer. The PB1 subunit contains the conserved polymerase domain and utilizes the primer for RNA elongation. In the phenotypic-assay-based drug discovery
effort, the 7-azaindole-based inhibitors target the PB2 cap-binding domain of the viral polymerase of influenza. Principally, 7-azaindole influenza inhibitors bind to the PB2 cap-binding domain and interfere with the replication and transcription of the viral RNA genome to exert pharmacological inhibitory activity.\(^{137}\)

The discovery of 7-azaindole-based anti-influenza compounds emerged from early work by Clark et al., who first identified a set of 7-azaindole analogs with considerable in vitro antiviral activity using branched DNA (bDNA) viral replication assay in cells and PB2 fluorescent polarization competition binding assay (Figure 35).\(^{138}\)

A 96-well bDNA assay detects the negative-strand RNA of the influenza A virus (A/PR/8/34 strain) using a set of oligonucleotides designed for the A/PR/8/34 nucleoprotein transcript.\(^{139}\) A cell-based antiviral assay was developed that depends on the multiplication of virus RNA in the infected cells with negative strand RNA levels being directly measured using the branched-chain DNA (bDNA) hybridization method. Cells were initially infected with the virus and incubated in the presence of test compound for approximately 20 h. Viral replication was quantified by determination of negative strand RNA levels by bDNA assay. The concentrations of the test compound resulting in viral RNA levels equal to that of 10% of the control wells were reported as EC\(_{90}\).

The binding affinity of compounds for the cap-binding domain of PB2 was determined using a competition binding fluorescent polarization (FP) assay. PB2 binding was examined using a 165 amino acid fragment of PB2 that had been identified as the cap-binding domain portion of the full-length protein. This PB2 domain was incubated with test compounds and with a 5'-FITC-labeled probe for 60 min at
room temperature to reach equilibrium. Values for probe-only wells were used as the background. PB2 $K_d$ values were determined by fitting the background-subtracted data to an equation for competitive displacement of a fluorescent probe.

The X-ray cocrystal structure of 96 complexed with the PB2 cap-binding domain (165 amino acid fragments) suggested that 96 and m7GTP (a known binder to PB2) displayed similar hydrogen-bonding interactions with the side-chain residues of PB2, engaging Glu361 and Lys376. The N-1 NH$_2$, O-6 of m7GTP and N-1 and N-7 elements of 96 were involved in H-bonding (Figure 36B).$^{138}$ The AI core of 96 was sandwiched between the aromatic side chains of His357 and Phe404, while the pyrimidine ring was $\pi$-stacked against Phe323. Although the dimethylalanyl moiety of 96 occupied the region to which the sugar phosphate group of m7GTP was bound, no direct polar interactions were observed with positively charged residues, such as Lys-339, Arg-355, and His-357. It was postulated that optimization of the amino substituent at the 4 position of the pyrimidine ring in 96 formed polar interactions with amino acids present around the sugar phosphate-binding region of m7GTP. To extend the structure toward the sugar phosphate-binding region of PB2, a series of compounds was synthesized by appending cycloalkyl, piperidine, diaminocylohexyl, and cyclohexyl carboxylate functionalities on the amino substituent at the 4 position of the pyrimidine ring in compound 96 (Figure 36). Consequently, the binding interactions of the cyclohexyl carboxylate-bearing [2,2,2]-bicyclooctane analog 99 with PB2 were similar to those of m7GTP (Figure 36C). In addition, the carboxylic group of 99 showed water-mediated interactions with the nitrogen of His357 and Gln406 as well as with the carbonyl group of Arg355. Overall, this study confirmed that 7-methylguanine and 7-azaindole occupy the same binding site in PB2. Furthermore, 99 demonstrated potent antiviral activity against a broad range of influenza type A strains in in vitro studies, Table 5. This study describes the emergence of several potent compounds; among them, 99 and the diaminocyclohexyl-based analog 102 were advanced to in vivo studies. Compound 99 provided 100% protection against influenza-induced death in mice when the test compound was administered at three doses (10, 3, or 1 mpk b.i.d. for 10 days) starting 48 h postinfection. However, 102 demonstrated only 75% survival when dosed at

![Figure 36. Comparison of the binding interactions of m7GTP and 7-azaindole analogs at the cap-binding domain of PB2. (A) m7GTP complexed with the PB2 cap-binding domain (PDB 4NCE). N-1,2-NH$_2$ and O-6 of the guanine ring are involved in binding interactions with Glu361 and Lys376. (B, C, D, and E) Compounds 96, 99, 100, and (R)-101 complexed with the PB2 cap-binding domain, respectively. Compounds 93 (PDB 4NCE), 99 (PDB 4P1U), 100 (PDB 4YD0), and (R)-101 (PDB 5JUR) occupied the same binding site of m7GTP and exhibited similar binding interactions with Glu361 and Lys376 residues; however, N-1 and N-7 of the AI ring were involved in H-bonding interactions.](https://doi.org/10.1021/acs.jmedchem.2c00444)
Table 5. In Vitro bDNA-Binding Assay and PB2 Fluorescence Competitive Binding Assay of Compounds 96, 99, and 102

| Structure and No. | bDNA EC99 (μM) | PB2 Kd (μM) |
|------------------|----------------|-------------|
| 96               | 1.13           | 0.10        |
| 99               | 0.004          | <0.003      |
| 102              | 0.012          | 0.40        |

*Concentration of the test compound resulting in viral RNA levels equal to 10% of the control wells is reported as the EC90. 
*Affinity of the cap-binding domain of the PB2 subunit as measured in a fluorescence polarization competition binding assay.

Table 6. In Vitro Cytotoxic Effect (CPE) Viral Assay and bDNA Assay of Compounds 99, 100, and 103

| Structure and No. | CPE IC50 (μM) | bDNA EC99 (μM) |
|------------------|---------------|----------------|
| 99               | 0.002         | 0.011          |
| 100              | 0.025         | 0.18           |
| 103              | 0.001         | 0.033          |

*MDCK cells were incubated with the test compounds and influenza A virus (A/PR/8/34 strain) for 72 h, and the concentration of the test compound resulting in 50% cell protection is reported as the IC50. 
*Concentration of the test compound resulting in viral RNA levels equal to 1% of the control wells is reported as the EC99.

30 mpk b.i.d for 10 days beginning 48 h postinfection. The standard drug oseltamivir, a neuroaminidase inhibitor when administered at the clinically relevant human equivalent dose (10 mpk b.i.d.) in the same animal model after 48 h of infection, did not provide any survival benefits.

Boyd and co-workers performed SAR studies on 99 by replacing the COOH group with different isosteres.140 One of the resulting compounds 100, which incorporates a tetrathiazole ring in place of the COOH moiety, exhibited 13- and 16-fold lower viral potency in the CPE and bDNA assays when compared to 99. However, the isoxazole analog 103 was equipotent to 99 in the CPE assay and only 3-fold less potent than 99 in the bDNA assay (Table 6).

The crystal structure data and computational studies suggested that 100 retained the H-bonding interactions with Lys376 and Glu361 of PB2 similar to 99. However, the isoxazole analog 103 was equipotent to 99 in the CPE assay and only 3-fold less potent than 99 in the bDNA assay (Table 6).

The crystal structure data and computational studies suggested that 100 retained the H-bonding interactions with Lys376 and Glu361 of PB2 similar to 99. However, the isoxazole analog 103 was equipotent to 99 in the CPE assay and only 3-fold less potent than 99 in the bDNA assay (Table 6).

Farmer and co-workers141 replaced the alanine dimethylamide side chain of 96 with an acyclic β-amino acid fragment bearing a nonpolar tert-butyl group (Figure 37) to form enantiomeric pairs. It was envisaged that attaching a nonpolar group at the β position may afford potential hydrophobic interactions with the aromatic amino acid residues Phe323, Phe325, and Phe404 present in the phosphate-binding region of PB2 while retaining the key-interactions, i.e., interaction of Phe323, and Phe404 present in the phosphate-binding region with Glu361 and Lys376. First, to validate this assumption, a docking analysis of compound (R)-101 into the active site of PB2 followed by superimposition of the X-ray structure of 96 complexed with PB2 was conducted (Figure 36). The overlay of 96 and (R)-101 at the cap-binding region of PB2 revealed good superimposition, and the tert-butyl group occupied the same area as the α-methyl dimethyl amide functionality. Encouraged by these findings, two enantiomeric pairs, namely, (R)-101, (S)-101 and (R)-104, (S)-104, were synthesized and evaluated for their binding affinity and antiviral potency. In support of the docking studies, the (R)-enantiomers (R)-101 and (R)-104 exhibited excellent binding affinity to PB2 (Kd = 0.003 and <0.003 μM, respectively) and antiviral potency (EC90 = 0.03 and 0.023 μM, respectively), whereas the (S)-enantiomers (S)-101 and (S)-104 were less potent (Figure 37). Furthermore, the X-ray co-crystal structure data for (R)-101 bound to PB2 suggested that N-1 and N-7 of the AI ring formed a H bond with Glu361 and Lys376 (Figure 36E), the AI ring was sandwiched between His357 and Phe404, while the pyrimidine ring was engaged in a π-stacking interaction with Phe323. The X-ray data also demonstrated that the hydrophobic pocket defined by the three phenylalanine residues Phe323, Phe325, and Phe404 could be utilized to forge stronger interactions with larger side chains (Figure 36E).

On the basis of the above findings, additional schemes were designed to develop a series of compounds having branched carbon chains (Figure 38).141 This study suggested that interactions with the hydrophobic pocket of PB2 could be
increased by the incorporation of hydrophobic groups on the side chain of the pyrimidine ring in AI analogs.

Among the analogs 105a–h, the spiro cyclobutane analog 105h exhibited the highest anti-influenza activity. The overall cellular potency and target affinity of (R)-101 (bDNA EC90 = 0.03 μM and PB2 Kd = 0.003) and 105h (bDNA EC90 = 0.01 μM and PB2 Kd ≤ 0.003) led to further study to assess the antiviral activity against a broad range of influenza type A strains. Interestingly, (R)-101 demonstrated potent antiviral activity against a broad range of influenza type A strains, including oseltamivir carboxylate-resistant isolates and the pandemic-causing H1N1 and H5N1 strains. In addition, the PK profiles of (R)-101 and 105h showed desirable iv and oral exposure in animal studies. (R)-101 provided complete protection at all three tested doses with all animals surviving (10, 30, and 60 mpk b.i.d for 10 days). Similarly, 105h also showed a complete survival benefit but only when dosed at 3, 10, and 30 mpk b.i.d beginning at 48 h postinfection. Thus, (R)-101 and 105h showed efficacy even giving it after 48 h in an influenza mouse model. However, the standard drug oseltamivir was devoid of efficacy in this mouse model when tested at the clinically relevant human equivalent dose (10 mpk b.i.d).

Notably, the primary metabolite observed for 102 (Table 5) in human hepatocytes was due to oxidation at the C-2 position of the 7-azaindole ring to form the 2-hydroxy-substituted metabolite 106 (Figure 39) mediated by the cytosolic enzyme aldehyde oxidase (AO), which catalyzes the oxidation of aza-heterocycles and aldehydes, amide hydrolysis, and diverse reductions.142

To bypass the AO-mediated metabolism of 7-azaindoles, C-2-substituted compounds were synthesized incorporating a
range of functionalities, including the addition of hydroxy methyl, methyl, cyclopropyl, carboxylic acid, oxime, and secondary alcohol groups (Figure 40B). This strategy was applied to 109, which has a pyrrolidine urea moiety and was found to be 12-fold more potent than 102 in the bDNA assay.142 In parallel, replacement of the C-2 hydroxy methyl group with a nitrogen atom to form an azaindazole ring (108a−h) was also envisioned as an approach to counter AO-mediated metabolism (Figure 40A).

Human liver cytosol stability studies have indicated that 109a, 109b, 109f, and 109g (Figure 40B) bypass AO-mediated metabolism at the C-2 position compared with compounds 102 and 107. The PK profiles of 107, 109g, and 108a in both mice and rats were favorable. Furthermore, the X-ray cocrystal structure of the complex of 109g bound to the PB2 subunit revealed that the interaction between the PB2 domain and 109g was similar to that of earlier molecules. An additional interaction was observed between the C-2 hydroxy methyl group through H bonds with Glu 361 and Arg 332 (Figure 41A).

McGowan and collaborators envisaged that 5,7-difluorindoles could be effective bioisosteres of the 7-azaindole ring system in which the fluorine atom at the 7 position could mimic an aromatic nitrogen atom and adopt a similar binding mode.143 In view of this, the 5,7-difluorindole analog 110 of 99 was synthesized (Figure 42). In addition, substituents with similar sizes or electronic characteristics to fluorine, such as methyl, cyano, and chloro groups at the C-5 position, were also prepared.

X-ray cocrystal studies of 110 (Figure 41B) indicated that the indole ring formed π-stacking interactions with Phe404 and His357 and ionic interactions with Glu361 and Lys376, similar to earlier 7-azaindole congeners. A few additional interactions were observed, including π−π stacking between the pyrimidine ring of 110 and Phe323 and an interaction between the positively charged guanidino group of Arg335 and the negatively charged carboxylate group of 110.

As anticipated, the C-7 fluorine atom of 110 interacted with Lys376, and the longer electronegative C−F bond length shifted 110 approximately 0.8 Å out of the binding pocket.

Thus, 110 adopted a slightly different conformation, allowing the carboxyl group to gain two additional ionic interactions with Arg335. This observation confirmed that 7-fluorindole could act as a bioisostere of 7-azaindole. Compound 110 showed excellent in vitro metabolic stability (Clint < 7.7 μL/min/mg protein) in human liver microsomes and was not susceptible to AO metabolism. The presence of fluorine atoms at the C-5 and C-7 positions of 110 may also be responsible for the enhanced metabolic stability.144 In vivo studies with 110 were performed at a dose of 30 mpk b.i.d. for 10 days in the mouse influenza model, where 75% survival was observed in animals administered the drug beginning at 48 h postinfection, whereas oseltamivir yielded only 38% survival at its therapeutic equivalent dose in mice (10 mpk b.i.d. for 10 days).

Given the above findings, we postulate the important structural features of a probable potent PB2 inhibitor, as shown in Figure 43. A coplanar heterocycle at the C-3 position of the AI core will be beneficial for π-stacking with a protein. A hydrophobic linker connecting a C-3-substituted heterocycle ring with another aromatic ring might provide better binding with PB2 connected through ionic and H-bonding interactions.

Taken together, AI and its isomers continue to serve as a core template with which to design new antiviral agents against HIV-1, HCV, DENV, RSV, and influenza. Replacing CH with N atoms in the AI/indole core was the most successful approach to attain multiparameter optimization in antiviral drug research. We created a summary of the reported bioisosteric replacements of AIs/indoles along with
the C-2 and C-3 substitution in antiviral drug discovery (Table 7).

**FUTURE PERSPECTIVES OF AI ANALOGUES IN REGULATING COVID-19-INDUCED CYTOKINE STORM OR HYPERINFLAMMATORY SYNDROME IN PATIENTS**

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), continues to spread globally despite unprecedented social isolation and restrictions resulting in widespread economic decline. More than 3.2 million people have been infected, and more than 230,000 of them have died. To date, no treatments have been definitively shown to be effective; however, a multipronged approach to mitigate transmission, morbidity, and mortality is ongoing. While upstream prevention strategies such as vaccination are ideal, these strategies are unlikely to be available in time to address current clinical need. Instead, fast tracking of drug development and repurposing of approved drugs has facilitated and expedited clinical trials that might hasten effective therapeutics. Many of these drugs act, at least in part, to directly limit viral replication. By contrast, the use of interleukin-6 (IL-6) inhibition might have benefits by controlling the pathological immune response to the virus. Here, we expand on the theoretical basis of IL-6 inhibition and propose potential benefits from other immunomodulators that could, in theory, prove more efficacious.

For the latter phase of convalescence, hospitalized patients with COVID-19 can develop a syndrome of dysregulated and systemic immune over activation described as a cytokine storm or hyperinflammatory syndrome that worsens acute respiratory distress syndrome and can lead to multisystem organ failure. The scarce systematic data available have shown an association between ferritin, lactate dehydrogenase, IL-6, IL-1, d-dimer, and C-reactive protein and severe disease. If this group can be identified before decompensation, early and aggressive immunomodulatory treatment might prevent the need for intubation and extracorporeal membrane oxygenation. To date, observational studies suggest a possible benefit, but results of placebo-controlled randomized clinical trials are not yet available. Given the methodological limitations of existing studies, more evidence is needed. With the rapidly expanding number of critically ill patients, there is an urgent need to identify multiple putative biological targets. While IL-6 inhibition attenuates key aspects of the cytokine cascade, we...
Figure 45. Proposed dual mechanism (anticytokine activity and inhibitors of host cell viral propagation) of the action of baricitinib (114) in COVID-19: (1) SARS-CoV-2 binds to host ACE 2 through the spike proteins (2) upon entry; SARS-CoV-2 induces clathrin-mediated endocytosis. AAK1 and GAK mediate the assembly of clathrin adapter proteins to form clathrin cages surrounding the virus before it is trafficked to endosomes (3) upon virus entry; activation of immune cells and release of chemokines (4) result in the recruitment of neutrophils, macrophages, NK cells, and T cells, which further damage alveolar epithelial cells via a process called the cytokine storm (5) baricitinib (114); AI analog has been proposed to inhibit 2 checkpoints, i.e., inhibit viral entry via AAK1- and GAK-mediated endocytosis and inhibit cytokine storms via JAK-STAT-dependent cytokine receptors.

posit other immune targets of inhibition to be considered and their potential to be more efficacious in the setting of COVID-19, specifically IL-1 inhibitors and Janus kinase (JAK) inhibitors.

Recently, molnupiravir (111) and paxlovid (112) have been approved by the FDA for emergency use as oral antiviral drugs against COVID-19 (Figure 44). However, the WHO has not yet recommended the use of these drugs for the treatment of COVID-19. The WHO recommended baricitinib, an oral drug, and sotrovimab, the monoclonal antibodies against COVID-19, yet recommended the use of these drugs for the treatment of COVID-19. However, the available treatment options for COVID-19 are essentially based on symptoms, and oxygen therapy is the main option for severely infected countries have not included it as a part of the therapeutic regimen against COVID-19. However, the available treatment options for COVID-19 are essentially based on symptoms, and oxygen therapy is the main option for severely infected patients. In cases of respiratory failure, artificial ventilation may be necessary.145 The symptomatic treatment modalities of COVID-19 are based mainly on three different mechanisms, i.e., reducing the viral load by drugs targeting viral entry, drugs inhibiting viral replication, and management of the hyper-inflammatory state by immunomodulating drugs.

Novel virus-based structural proteins have been identified as drug targets in COVID-19, such as the nucleocapsid N protein, spike S glycoprotein, and several virus-based nonstructural proteins. Similarly, host-based targets, such as the ACE2 receptor, AAK1/GAK, JAK, transmembrane serine protease 2, furin, cathepsin L, phosphatidylinositol 3-phosphate 5-kinase, and two-pore channels, have also been identified as potential host targets.146,147

More than 100 drugs from diverse therapeutic classes were proposed for their potential to be repurposed for COVID-19 by taking advantage of current information on their safety pharmacology to enable rapid clinical trials and regulatory review.148 Recently, the potential role of AI-based JAK inhibitors 114 and 116 (Figure 44) in combating cytokine storms or hyperinflammatory syndrome in COVID-19 patients has been explored. Hyperinflammatory syndrome is one of the primary causes of multiple organ failure and death.149,150 ACE2 and CD147 (cluster of differentiation 147) are the two important receptors that are thought to be involved in SARS-CoV-2 invasion and dissemination into the host cells.151 In the early stages of SARS-CoV-2 infection, the positive role of the transmembrane protein ACE2 has been confirmed in alveolar epithelial cells in conjunction with the cellular protease TMPRSS2.152 ACE2 binds to the spike proteins on the capsid of SARS-CoV-2 (Figure 45), which subsequently initiates clathrin-dependent endocytosis of SARS-CoV-2.153

Following SARS-CoV-2 infection, a reciprocal state tends to be established in which downregulation of the renin-angiotensin system affords a natural protective effect along with an upregulation of proinflammatory cytokines.153 From the clinical data, higher serum levels of many cytokines, such as IL 6, IL 2, IL-1b, IL-8, IL-17, IFN-g, TNF-a, IP 10, MCP-1, IL-10, and IL-4, have been documented in COVID-19 patients.154

Therefore, targeting JAK-STAT-dependent signaling with JAK inhibitors to reduce the production of IL-6 and other cytokines was thought to be a direct approach to mitigate the cytokine storms associated with COVID-19 (Figure 45). Five clinical trials were explored which were designed to address the safety and efficacy of 114 in COVID-19-infected patients at clinical doses of 2–4 mg daily for 7–14 days.149 Moreover, 114 has been reported to block the intracellular trafficking of SARS-CoV-2 virus, which is regulated by clathrin-associated host adaptor proteins controlled by AAK1 and cyclin G-related kinases (GAK).155 Molecule 114 binds with high affinity to AAK1 (17 nM), JAK1 (6 nM), and JAK2 (6 nM), affording a potential advantage in countering SARS-CoV-2 COVID-19 infectivity.156 In addition, 114 has been reported for treating inflammatory conditions, such as rheumatoid arthritis and myelofibrosis. Recently, in 2021, the FDA issued warnings for the use of the JAK inhibitors against chronic inflammatory conditions. The FDA advisory highlighted the increased risk of heart-related events such as heart attack, stroke, blood clots, and death of few patients. There is higher risk involved with
these drugs for current or past smoking patients or those who had a heart attack. It was advised that all of the clinical health care professionals must consider the benefits and risks for the individual patient prior to initiating or continuing therapy using JAK inhibitors against COVID-19 infection.

Next, it was proposed that 114 may be effective against the elevated levels of cytokines at its therapeutic dose in COVID-19 patients.156,157 Fine tuning of the selectivity of AI toward JAK and AAK1 may yield multitargeted molecules against COVID-19. JAK-targeting compounds may be developed as anticytokine medicines against various inflammation-associated diseases, including COVID-19. In contrast, compounds selective for AAK1 can be established as early-phase medication agents in SARS-CoV-2 and other viral infections.

Virus-based nonstructural proteins (nsp)s serve an essential function in the lifecycle of SARS-CoV-2.158 The eukaryotic mRNA 5′-cap structure is considered necessary for RNA stability by conferring a molecular signature for self- or nonself-mRNA distinction.159 To escape innate host immunity, the 5′-end of the viral RNA gets modified by forming an $m^\prime$GTP cap and a C2′-O methyl cap on the adjacent nucleotide (Figure 46).160 In eukaryotes, 5′ capping is introduced on newly transcribed host mRNA already present within the nucleus to which SARS-CoV-2 does not have access. To overcome this inaccessibility, the virus has evolved to synthesize its own capsulating machinery in the cytoplasm.

In particular, nsp14 and nsp16 are responsible for methylation of the guanine of the GTP and the C2′ hydroxyl group of the adjacent nucleotide (Figure 46). In SARS-CoV-2, nsp10 in conjunction with nsp14 methylates the 5′-end of virally encoded mRNAs to mimic cellular mRNAs, thus protecting the virus from host innate immune restriction. nsp16 seems to be a very promising molecular target for drug discovery, and the crystal structure of SARS-CoV-2 nsp10 and nsp16 in complex with the purine analogs sinefungin (pan-MTase inhibitor) and SAM (natural methyl donor) provides a strong foundation for structure-based inhibitor design for COVID-19 (Figure 47).158,160 The binding pattern of the 7-azaindole analogs 96, 99, (R)-101, 103, 109, and 110 includes N-1 and N-7 of AI, which undergo H-bonding interactions similar to the purine ring of $m^\prime$GTP (N-1, 2-N1, 2-H2, O-6) and occupy the purine-binding domain at the Pb2 cap-binding site (Figures 36 and 41). These features confirm the bioisostere nature of 7-azaindole with purine in the PB2 binding pocket. The antifulfluenza activity of 7-azaindole results from its inhibition of the influenza virus cap-snatching mechanism. SARS-CoV-2 also exhibits a similar capping mechanism for its RNA stability with the help of nsp14 and nsp16.160

Therefore, we believe that structure-guided optimization to form 7-azaindole-based nucleosides could generate promising scaffolds. Alternately, the incorporation of carbocyclic/heterocyclic mimics of sugar amino acids at the C-3, C-4, or C-5 position of 7-azaindole could be a rational structural design strategy to develop AI-based inhibitors to combat COVID-19 and other viral diseases.

CONCLUSION

Our in-depth and critical survey of a decade of work on AIs and their analogs reinforces the perspectives of remarkably successful antiviral agents against a wide spectrum of RNA viruses. It is thus imperative to modify AI-containing compounds to attenuate the H-bonding capacity, physiological profile, pharmacological characteristics, and physicochemical
Table 7. Summary of Bioisosteric Replacements of AIs/Indoles and C-2 and C-3 Substitutions on AIs in the Design of Antiviral Agents

| Transformation | Example | Pharmacological profile |
|---------------|---------|-------------------------|
|               | ![Image](image1.png) | 1) Readily hydrolyzed and stable <br>2) Low blood clearance in humans <br>3) Moderate bioavailability in humans and t½ = 4.5 h <br>4) No inhibition of CYP isoforms, HCV polymerase and Mg-dependent protein kinase <br>5) Attenuated clearance rates in human hepatocytes |
| C-3 C-5 substitution | ![Image](image2.png) | 1) 300-fold improvement in antiviral potency, clearance in both human liver microsomes and human hepatocytes <br>2) Improved \textit{in vitro} permeability <br>3) Reduction in glucuronidation rates \textit{in vitro} <br>4) Comparable \textit{in vivo} human PK parameters |
| CH=N | ![Image](image3.png) | 1) Similar potent activity for 49-50, but variable PK profile <br>2) N at C-5 (51) yielded refined n-stacking, thus increasing the solubility and lung distribution of the compounds |
| CH=N | ![Image](image4.png) | N at C-6 in 52 yielded lower potency (8-10-fold) |
| CH=N | ![Image](image5.png) | 1) Substantial permeability and similar metabolic stability in rats <br>2) Less protein bound, excellent plasma exposure, moderate clearance after iv dosing and low distribution to the lung in rats |
| CH=N | ![Image](image6.png) | 1) Beneficial for both permeability and potency, acceptable metabolic stability in rats <br>2) Less protein binding, moderate plasma exposure, high clearance, and well distributed to the lung in rats |
| CH=N | ![Image](image7.png) | 1) Decreased lipophilicity (enhanced solubility), and a decrease in antiviral potency <br>2) Effective in attenuating Pgp recognition |

H-bonding interaction of N-1 and N-7 of 99 and N-1,2-N1,2-H5, O-6 of the mGTP ring are similar at the P92 binding site.
properties of AIs to obtain lead compounds against different viruses.

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**Notes**

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Urvashi received her B.Sc. degree in 2011 and M.Sc. degree in Organic Chemistry from Delhi University in 2013. She joined the Special Centre for Molecular Medicine, Jawaharlal Nehru University as a junior research fellow for a few months. She received her Ph.D. degree from Delhi University in 2021, and her doctoral thesis title was “Transition-Metal-Catalyzed C–C/C–S Coupling and C–N Bond Formation: Synthesis of N-Heterocyclic Compounds and their Biological Evaluation”. Briefly, her research interests include finding a flexible route to access heterocyclic frameworks (C–C/C–S/C–N) with challenging substitution patterns using transition-metal-catalyzed cyclization, condensation, coupling, and addition reactions and to evaluate them for medicinal purposes.

J. B. Senthil Kumar obtained his Ph.D. degree in Medicinal Chemistry in 2014 from the University of Delhi, North Campus. The main focus of his Ph.D. thesis was to identify a newer class of dopamine agonists by structural modification of a natural product ergoline ring to obtain promising compounds with in vivo efficacy animal models of Parkinsonism. Currently, he is working as a DS Kothari Postdoctoral Fellow (UGC) at the Special Centre for Molecular Medicine, Jawaharlal Nehru University. His research interest includes the design and synthesis of neuroprotective agents for the mitigation of oxidative stress associated with neurological disorders.

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### ABBREVIATIONS USED

AAK1, adaptor-associated kinase 1; ACE2, angiotensin-converting enzyme 2; ADCC, antibody-dependent cellular cytotoxicity; ADME, absorption, distribution, metabolism, and excretion; AI, azaindole; ALRT, acute lower respiratory tract infection; AMLV, amphotropic murine leukemia virus; AO, aldehyde oxidase; BBB, blood–brain barrier; BCS, Biopharmaceutics Classification System; bDNA, branched DNA; BIF, bioisostere factor; BMP2K, bone morphogenetic protein 2 inducible kinase; BMS, Bristol Myers Squibb; CART, combination antiretroviral therapy; CD147, cluster of differentiation 147; CDC, complement-dependend cytotoxicity; cDNA, complementary DNA; CNS, central nervous system; CPE, cellular protection assay; CPR, cytopathic effect; CYP3A4, cytochrome P450 3A4; DENV, dengue virus; DNP, 1,3-dinitrophenyl; DTG, dolutegravir; EEF1A1, elongation factor 1A-1; EMD, expression microarray database; ERK, extracellular signal-regulated kinase; EZH2, enhancer of zeste homolog 2; FGFs, fibroblast growth factors; FCS, fetal calf serum; FGF2, fibroblast growth factor 2; FGF-18, fibroblast growth factor 18; FFU, focus forming unit; FMDV, foot-and-mouth disease virus; FMT, fecal microbiota transplantation; GABA, γ-aminobutyric acid; GAGs, glycosaminoglycans; GAK, cyclin G-associated kinase; GPCR, G protein-coupled receptor; GSK3β, glycogen synthase kinase 3β; GTP, guanosine triphosphate; HCV, hepatitis C virus; hERG, Human Ether-a-go-go-Related Gene; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-lymphotropic virus; HSP, heat shock protein; IDO, indoleamine 2,3-dioxygenase; IGF, insulin-like growth factor; IL, interleukin; IN, integrase; IRF-3, interferon regulatory factor-3; JAK, Janus kinase; JAK-STAT, Janus kinase signal transduction and transcription; LIF, leukemia inhibitory factor; mAb, monoclonal antibody; mGTP, 7-methylguanosine 5′-triphosphate; MLM, mouse liver microsomes; MTases, methyl transferases; NAKs, NUMB-associated kinases family; nps, nonstructural proteins; NIAID, National Institute of Allergy and Infectious Disease; NRRTIs, non-nucleoside reverse transcriptase inhibitors; NS4B, nonstructural membrane bound protein; PBMC, peripheral blood mononuclear cells; PIs, protease inhibitors; PI3Kα, phosphoinositide-3-kinase γ inhibitors; PK, pharmacokinetics; RAL, raltegravir; RdRp, RNA-dependent RNA polymerase; RSV, respiratory syncytial virus; RT, reverse transcriptase; SAM, S-adenosyl methionine; SAR, structure–activity relationship; SARS, severe acute respiratory syndrome; SFV, Semliki Forest virus; SIN, Sindbis virus; STK16, serine/threonine protein kinase 16; TM, transmembrane anchor; WEEV, Western equine encephalitis virus.

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