New Pyrazine Conjugates: Synthesis, Computational Studies, and Antiviral Properties against SARS-CoV-2

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Currently, limited therapeutic options are available for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). We have developed a set of pyrazine-based small molecules. A series of pyrazine conjugates was synthesized by microwave-assisted click chemistry and benzotriazole chemistry. All the synthesized conjugates were screened against the SAR-CoV-2 virus and their cytotoxicity was determined. Computational studies were carried out to validate the biological data. Some of the pyrazine-triazole conjugates (5d–g) and (5)-N-(1-(benzo[d]thiazol-2-yl)-2-phenylethyl)pyrazine-2-carboxamide 12i show significant potency against SARS-CoV-2 among the synthesized conjugates. The selectivity index (SI) of potent conjugates indicates significant efficacy compared to the reference drug (Favipiravir).

Introduction

An unknown viral infection originated from China in December 2019 and rapidly spread across the world.[1–3] The World Health Organization (WHO) identified the virus as the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) on Feb. 11, 2020 and named it coronavirus disease 2019 (COVID-19). In March 2020, WHO declared the coronavirus outbreak as a global pandemic.[4] As of July 2021, over 200 million people have been infected, and more than 4.2 million people worldwide have died from the coronavirus. Although the world has survived numerous pandemics in the past, COVID-19 is an unprecedented global health challenge that has greatly impacted our lives and the socioeconomic structure of the world.

Very few antiviral drugs are available for the treatment of COVID-19 and most of them are ineffective. Of the few vaccines that were given emergency approval by the FDA, some are showing adverse side effects. The pandemic has spurred research into the development of new drugs and exploration of the existing antiviral drugs against COVID-19. By using efficient drug development to control the global pandemic and reducing the time from synthesis to FDA approval, the drug repurposing approach would be the most accessible and pragmatic pathway. Many drugs were considered for this subject. Favipiravir is a drug that exhibits promising response with limitations such as cardiovascular, endocrine, gastrointestinal, and hepatic toxicity. As such, that drug may need further investigational studies (Figure 1).[5,6] Recently, several countries approved Favipiravir for emergency use to control the pandemic. As of April 2021; there are 33 studies registered on clinicaltrials.gov to assess the utilization of this drug in the management of COVID-19.[7]

New drug development process is challenging, time-consuming and expensive. Among various rational drug design strategies, molecular hybridization of bioactive moieties is a powerful and attractive approach because of several advantages such as a) increasing desired pharmacological activity; b) allowing for better selectivity towards the target; c) improving interaction with multiple pharmacological sites and d) decreasing possible cytotoxicity. Our continuous research mission is to actively design and develop potential drug candidates for targeted diseases by this molecular hybridization approach.[8–13]

Recently, we have reported a set of quinoline-triazole conjugates and some of them are showing promising activity against SARS-CoV-2.[14] In continuation of our drug development approach, we have utilized “click’ chemistry” and “benzotriazole chemistry” in synthesizing new pyrazine-based conjugates as...
potential antiviral drug candidates for SARS-CoV-2. We have also synthesized another set of hybrid conjugate of pyrazine and benzothiazole using amino acids as linkers. We selected the pyrazine scaffold from the repurposing drug (Favipiravir) and triazole moiety because of their importance in the drug development process and because they are well-known for diversified biological properties. Benzothiazole is also known for distinct biological properties including antitumor, anti-inflammatory, antimicrobial, antitrypanosomal, anticonvulsant, and antituberculosis activities.

Results and Discussion

An efficient strategy was developed to synthesize the desired pyrazine-triazole conjugates 5a–g from pyrazine-based alkyne 3 with substituted aromatic azides 4 by adopting the well-established Cu-mediated click chemistry (Scheme 1). The precursor alkynes 3 were synthesized by treating a solution of pyrazinoic acid 1 in DMF with propargyl bromide 2 to obtain the alkyne component under the basic environment of potassium carbonate (K₂CO₃) at room temperature. The azides of aromatic amines 4a–g were synthesized following the previously reported procedure. To diversify the pool of favipiravir-based compounds, we activated the carboxylic acid of pyrazinoic acid with benzotriazole and the benzotriazolide treated with various amino acids to get the pyrazinoic acid-amino acid conjugates. The conjugates were further treated with 2-amino thiophenol under microwave irradiation to obtain another set of conjugates 12a–k containing pyrazine and benzothiazole moieties. We have also tried to synthesize the same set of conjugates 12a–k using benzotriazole chemistry and in both routes, we observed similar results (Scheme 2).

We investigate an alternate route to synthesize the conjugates 12a–k using Boc chemistry and benzotriazole chemistry to avoid the use of excessive thionyl chloride by following the synthetic route described in Scheme 3. In this route, we observed that the overall yield is lower than the previous route.

To understand the role of amino acids in the conjugates, we have synthesized the conjugate containing pyrazine and benzothiazole without amino acid linker following a similar reaction condition (Scheme 4).

Antiviral properties

Antiviral properties of compounds 5a–g, 12a–k, and 16 were determined against SARS-CoV-2 utilizing the standard protocol. Favipiravir was used as a standard reference.
(positive control) for the study. Cytotoxic properties were also determined against the normal cell line VERO-E6 by the standard MTT technique to evaluate the therapeutical/selectivity index of the tested compounds. Table 1 (Figure 2) reveals that compound 5e is superior among all the tested analogs with selectivity index 2.71 folds relative to the standard reference used (Favipiravir) due to its higher potency (IC50) against viral cells and lower cytotoxicity (CC50) against normal cells (IC50 = 0.477, 1.382; CC50 = 4.916, 5.262 mM for 5e and Favipiravir, respectively). Compound 12i also shows a similar observation (IC50 = 0.3638; CC50 = 1.396 mM) with slightly higher selectivity index that of Favipiravir (SI = 3.837). Compound 5f exhibits a close therapeutical index to that of the standard reference (SI = 3.685). Although compound 5d reveals the highest potency against SARS-CoV-2 among all the tested compounds its lower selectivity index than Favipiravir (IC50 = 0.120, CC50 = 0.378 mM; SI = 3.150) hindered its biological viability.

Based on the observed results, few SAR (structure-activity relationships) can be assigned. The methoxy group attached at the para-position of the phenyl ring at the N-1 of triazolyl heterocycle seems essential for optimizing an effective antiviral agent comparable with the halogen substituent (fluorine or chlorine) as shown in compounds 5a/5b/5d. However, an opposite rule was exhibited for the ortho substitution. Where the o-chlorophenyl containing agent is of an enhanced antiviral property than the o-methoxyphenyl analog and better selectivity index as exhibited in pairs 5f/5g (IC50 = 0.952, 1.079; CC50 = 3.508, 2.308 mM; SI = 3.685, 2.139 for 5f and 5g, respectively).

It has also been noticed that the unsubstituted methylene connecting the benzothiazolyl and pyrazinecarboxamide heterocycles are of higher antiviral properties relative to the substituted methylene containing-analogs as shown in compound 12a (IC50 = 0.2064 mM). Utilization of phenylalanine for constructing pyrazine-benzothiazole conjugate afforded a potent antiviral active agent with enhanced therapeutical index than the tryptophan amino acid as shown in pairs 12i/12h (IC50 = 0.3638, 2.993; CC50 = 1.396, 1.142 mM; SI = 3.837, 0.382 for 12i and 12h, respectively).

Compounds 5a–g have ester linkage and 12a–k have amide linkage. Several triazole-containing bioactive molecules were reported for in-vitro and in-vivo activities in the literature.
which are having ester linkage.\textsuperscript{[29–31]} We believe our synthesized conjugates need further investigation to determine the \textit{in-vivo} stability.
QSAR study

QSAR is an important computational technique widely used in medicinal chemical studies for determining the parameters essential for bio-observations. It is usually used to predict/calculate the bio-properties via mathematical equations in terms of descriptors (physicochemical parameters).\[30\] Robust three descriptor model ($R^2 = 0.872$, $R^\text{cvOO} = 0.818$, $R^\text{cvMO} = 0.783$) was obtained describing the antiviral properties of the tested conjugates (Supporting Information Figure S1, Tables S1–S3). The QSAR model covers a wide range of antiviral properties ($IC_{50} = 12 \text{ mM}$; due to $IC_{\text{observed}} = 0.120–12.223$, $IC_{\text{predicted}} = 0.227–12.137$).

The number of fluorine atoms is a constitutional descriptor with the highest Student $t$-criterion value among the other model’s descriptors ($t = 7.627$) with a positive coefficient factor (8.852). This is an indication of the low antiviral properties of the compound possessing a high mathematical descriptor value. The appearance of this descriptor with a high $t$-value supports the previously mentioned SAR due to the low antiviral properties of compounds possessing p-fluorophenyl ring relative to the p-methoxyphenyl system as exhibited in pairs 5a/5d (descriptor value $\approx 1$, 0; for 5a and 5d, respectively).

The maximum bond order of atom C is a semi-empirical descriptor with a negative coefficient value ($-44.3495$). This is why the conjugate with a high mathematical descriptor value optimizes potent effective agent as shown in pairs 5a/5e (descriptor value $= 1.778$, 1.854 corresponding to predicted $IC_{50} = 12.137$, 0.227 mM for 5a and 5e, respectively). Mulliken bond orders can be calculated by Equation (1).\[32\]

$$P_{AB} = \sum c_{i\alpha} c_{i\beta}^{\ast}$$

(1)

Where, $n_i$ stands for the occupation number of the $i_{th}$ molecular orbitals. The $c_{i\alpha}$, $c_{i\beta}$ are the molecular orbital coefficients for the atomic orbitals $\alpha$ and $\beta$.

The HACA-1/TMSA is a charge-related descriptor that also negatively participated in the QSAR model ($-113.9$). Again the higher mathematical descriptor value estimates higher antiviral potency as shown in pairs 5f and 5g (descriptor value $= 0.01139$, 0.01081 corresponding to predicted $IC_{50} = 0.439$, 1.867 mM for 5f and 5g, respectively). The appearance of HACA-1 (hydrogen bonding acceptor ability) as an important descriptor controlling the observed bio-properties, also supports the attained SAR due to the role of the methoxy group relative to the halogen substituent attached to the phenyl ring at the para-position in enhancing the antiviral properties. The fractional hydrogen bonding acceptor ability of a molecule (FHACA-1) can be calculated by Equation (2).\[32\]

$$FHACA = \frac{HACA - 1}{TMSA}$$

(2)

Where, HACA-1 and TMSA stand for the hydrogen bonding acceptor ability and total molecular surface area, respectively.

The advantage of the QSAR model achieved is supported by the statistically parameters $F$ and $s^2$ (Fisher significance and standard deviation $= 29.416$, 1.615, for $F$ and $s^2$ respectively) in addition to the internal validation values ($R^\text{cvOO} = 0.818$, $R^\text{cvMO} = 0.783$), also add good support for the QSAR model that can be utilized in a future study for predicting promising antiviral hits.

Molecular modeling study

RdRp (RNA-dependent RNA polymerase) is an important enzyme controlling the life cycle of many viruses including coronavirus. This is due to its circular role in the transcription and replication of viral RNA. This is why the RdRp inhibitors are recognized as druggable antiviral agents.\[33\] Favipiravir is originally developed as an anti-Flu viral agent. Due to its RdRp inhibitory properties, it is considered for clinical trial against SARS-CoV-2.\[34\] The synthesized agents revealing variable anti-SARS-CoV-2 properties were considered for molecular modeling (Discovery Studio 2.5 software, standard CDOCKER technique, PDB: 7CTT) for validating the bio-observations.\[35\]

Compound 5d, the most potent synthesized agent ($IC_{50} = 0.120 \text{ mM}$), shows hydrogen bonding interactions with ARG555, CY5622, and LYS798 (the same amino acids revealing interactions with the co-crystalized ligand) in addition to three hydrogen bindings with LYS621 due to the pyrazinyl N and CO interactions. The $\pi$-cation interaction of the pyrazinyl heterocycle with the LYS798 also supports the tested agent in the protein active site affording a higher docking score relative to Favipiravir (docking score $= -40.960$, $-28.665 \text{ kcal mol}^{-1}$ for 5d and Favipiravir, respectively) (Supporting Information Figure S2).

Compounds 12a and 12i which are also potent anti-SARS-Co-V2 agents ($IC_{50} = 0.2064$, 0.3638 mM for 12a and 12i, respectively) show hydrogen bonding with ARG555 due to the amido CO interaction in similar behavior to Favipiravir. The additional hydrogen bonding of amido NH with ASP760 supports the tested agents in the protein active site giving rise to the enhanced docking score values (docking score $= -34.02$, $-41.81$ for 12a and 12i, respectively). On the other hand, compound 5d reveals hydrogen bonding interactions with CY5622 and LYS798, which are important amino acids of protein active site revealing interaction with the co-crystallized agent. The three hydrogen bindings of the pyrazinyl N and CO with the LYS621 enhance the docking score and explain the antiviral potency observed ($IC_{50} = 0.477 \text{ mM}$, docking score $= -42.578 \text{ kcal mol}^{-1}$ for 5d). Generally, the molecular modeling studies in the PDB: 7CTT support the observed anti-viral properties, considering that the slight divisions between the in-vitro antiviral and docking score values are due to the difference in the applied conditions of the experimental and computational techniques.
ADME study

ADME (absorption, distribution, metabolism, and excretion) properties were determined by using a computational tool (Discovery Studio 2.5 software).\textsuperscript{10} We believe these properties are important in addition to the pharmacological data. From the observed results (Supporting Information Table S4) it has been noticed that most of the tested agents show good aqueous solubility levels. Additionally, all the tested agents reveal good intestinal absorption. Plasma protein binding of all the promising synthesized antiviral agents (5d–g and 12i) are at the level of 90–95%.

Conclusion

In summary, we have designed and synthesized two sets of pyrazine-based conjugates by optimized facile reaction conditions in good yields. Some of the synthesized conjugates (5d–g and 12i) showed promising antiviral properties against SARS-CoV2. The selectivity index (SI) of the potent compounds 5d showed promising antiviral data are encouraging. The computational studies adopted SARS-CoV2. The selectivity index (SI) of the potent compounds 5d showed promising antiviral properties against SARS-CoV-2 and cytoxic data are encouraging. The computational studies adopted QSAR and molecular modeling studies support the observed biological properties. Calculated ADME properties are interesting and promising. The initial investigation data and computational studies could be further considered as important tools to develop new potential drug candidates for COVID-19.

Experimental Section

Melting points were determined on a capillary tube melting point apparatus equipped with a digital thermometer Stuart SMP30 (Stuart, Cole-Parmer Instrument Co. Ltd, Staffordshire, UK). NMR spectra were recorded in CDCl3 or DMSO-d6, on a Bruker NMR spectrometer (Bruker Corporation, MA, USA) operating at 500 MHz for 1H with tetramethylsilane (TMS) as an internal standard and 125 MHz for 13C. HPLC high-resolution mass spectrometry (HRMS) analyses were performed on reverse phase gradient using Agilent 1200 series binary pump (G1312B; Agilent, CA, USA), waters XTerra column (2 × 4 mm) using 0.2% acetic acid in H2O (1.4 equiv.) was added.

Preparation of prop-2-yn-1-yl pyrazine-2-carboxylate 3

We synthesized compound 3 by modifying a literature-reported procedure.\textsuperscript{16} To a suspension of pyrazinoic acid 1 (1 equiv.) in N, N-dimethylformamide (DMF; 10 mL), K2CO3 (1.4 equiv.) was added. After stirring for 30 min, propargyl bromide (1 equiv.) was added and the reaction mixture was stirred overnight. The crude mixture was diluted with water then, extracted with EtOAc (3 × 30 mL) and the combined organic layer was dried over sodium sulfate, evaporated under reduced pressure and the crude residue was purified by column chromatography.

Preparation of pyrazinoic-triazole hybrids (5a–g)

To a solution of prop-2-yn-1-yl pyrazine-2-carboxylate 3 (1 mmol) in t-butanol/H2O (2: 1 v/v) (3 mL), CuSO4 and sodium D-isoascorbate monohydrate was added at room temperature. To this mixture, ary azide 4 (1.5 mmol) was added and the reaction mixture was stirred overnight. The crude mixture was diluted with water then, extracted with EtOAc (3 × 30 mL) and the combined organic layer was dried over sodium sulfate, evaporated under vacuum, and purified through column chromatography to give the pure pyrazinoic-triazole derivatives 5a–g in good yields.

(1-(4-Fluorophenyl)-1H-1,2,3-triazol-4-yl)methyl pyrazine-2-carboxylate (5a)

White microcrystals, m.p. 130–132°C, yield 50% (0.45 g). IR: νmax/cm−1 3140, 2931, 1732, 1517, 1409, 1286, 992, 945, 834, 771. 1H NMR (500 MHz, CDCl3) δ: 9.32 (s, 1H), 8.76 (s, 1H), 8.71 (s, 1H), 8.15 (s, 1H), 7.67 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 5.64 (s, 2H). 13C NMR (125 MHz, DMSO-d6) δ: 158.8, 156.8, 143.2, 141.8, 138.3, 138.2, 128.3, 117.9, 117.9, 112.1, 111.9, 54.3. HRMS m/z for: C10H8FN2O [M + H]+ Calcd. 238.0819. Found: 238.0821.

(1-(4-Chlorophenyl)-1H-1,2,3-triazol-4-yl) methyl pyrazine-2-carboxylate (5b)

White microcrystals, m.p. 135–138°C, yield 36% (0.32 g). IR: νmax/cm−1 3140, 2931, 1712, 1501, 1443, 1244, 983, 936, 825, 778. 1H NMR (500 MHz, CDCl3) δ: 9.32 (s, 1H), 8.76 (s, 1H), 8.71 (s, 1H), 8.12 (s, 1H), 7.85–7.47 (m, 2H), 7.35–6.80 (m, 2H), 5.65 (s, 2H). 13C NMR (125 MHz, DMSO-d6) δ: 159.1, 143.2, 141.7, 139.9, 138.3, 138.2, 130.5, 130.1, 125.3, 117.9, 117.1, 117.0, 54.3. HRMS m/z for: C10H7ClN2O [M + H]+ Calcd. 263.0523. Found: 263.0529.

(1-(p-Tolyl)-1H-1,2,3-triazol-4-yl) methyl pyrazine-2-carboxylate (5c)

White microcrystals, m.p. 112–114°C, yield 45% (0.41 g). IR: νmax/cm−1 3140, 2931, 1708, 1519, 1447, 1423, 938, 816, 776. 1H NMR (500 MHz, CDCl3) δ: 9.32 (s, 1H), 8.76 (s, 1H), 8.72 (s, 1H), 8.13 (s, 1H), 7.58 (d, J = 8.3 Hz, 2H), 7.26 (d, J = 8.2 Hz, 2H), 7.35 (s, 2H), 2.40 (s, 3H). 13C NMR (125 MHz, CDCl3) δ: 164.09, 148.12, 146.67, 144.78, 143.29, 142.75, 139.42, 134.75, 131.57, 130.5, 122.9, 121.0, 121.5, 59.39, 21.32. HRMS m/z for: C10H10ClN2O [M + H]+ Calcd. 296.1069. Found: 296.1073.

(1-(4-Methoxyphenyl)-1H-1,2,3-triazol-4-yl) methyl pyrazine-2-carboxylate (5d)

White microcrystals, m.p. 110–112°C, yield 36% (0.32 g). IR: νmax/cm−1 3140, 2931, 1721, 1518, 1409, 1255, 904, 841, 830, 774. 1H NMR (500 MHz, CDCl3) δ: 9.29 (s, 1H), 8.73 (s, 1H), 8.69 (s, 1H), 8.07 (s, 1H), 7.64–7.51 (m, 2H), 6.96–6.93 (m, 2H), 5.62 (s, 2H), 3.82 (s, 3H). 13C NMR (125 MHz, CDCl3) δ: 151.3, 148.9, 148.2, 146.7, 130.5, 126.4, 126.3, 125.7, 121.5, 112.4, 59.5, 56.2. HRMS m/z for: C10H12ClN2O [M + H]+ Calcd. 312.1018. Found: 312.1019.

(1-(4-Nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl pyrazine-2-carboxylate (5e)

White microcrystals, m.p. 172–174°C, yield 34% (0.21 g). IR: νmax/cm−1 3145, 2931, 1710, 1505, 1415, 1299, 958, 843, 795, 758. 1H NMR (500 MHz, DMSO-d6) δ: 9.31 (s, 1H), 8.76 (d, J = 2.1 Hz, 1H), 8.75 (s, 1H), 8.40 (d, J = 9.0 Hz, 2H), 8.30 (s, 1H), 7.97 (d, J = 9.0 Hz, 2H), 5.67
White microcrystals, m.p. 120–122 °C, yield 27% (0.24 g). IR: ν\textsubscript{max} \text{cm}^{-1} 3145, 2931, 1710, 1505, 1415, 1299, 958, 843, 795, 758. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ: 9.37 (s, 1H), 8.77 (d, J = 2.4 Hz, 1H), 8.60 (s, 1H), 8.54 (d, J = 8.8 Hz, 1H), 8.26 (d, J = 8.2 Hz, 1H), 8.14 (d, J = 8.3 Hz, 1H), 7.66 (t, J = 7.7 Hz, 1H), 7.52 (t, J = 7.7 Hz, 1H), 6.15 (dd, J = 9.0, 5.1 Hz, 1H), 2.44–2.40 (m, 1H), 1.16 (d, J = 6.8 Hz, 3H), 1.07 (d, J = 6.9 Hz, 3H). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) δ: 164.9, 162.4, 174.8, 147.6, 114.7, 143.9, 143.0, 142.8, 135.1, 128.1, 127.4, 124.5, 119.8, 116.6, 66.1, 34.4, 17.7, 17.4. HRMS m/z for C\textsubscript{13}H\textsubscript{12}N\textsubscript{3}O\textsubscript{3} (M + Na\textsuperscript{+}) Calcd. 312.3910. Found: 312.3915.

Preparation of pyrazinoic acid-aminoacyl benzotriazoles 10a–k

A dried heavy-walled Pyrex tube containing a small stir bar was charged with benzotriazole intermediate 10a-k (0.7 mM) and 2-aminothiophenol 11 (0.7 mM) dissolved in THF (3 mL). The reaction mixture was exposed to microwave irradiation (50 W) at a temperature of 100 °C for 2 h. Each mixture was allowed to cool through an inbuilt system until the temperature had fallen below 30 °C (ca. 10 min). The reaction mixture was quenched with ice-cold water and the solid obtained was filtered and washed with Na\textsubscript{2}CO\textsubscript{3} solution (10%) and water to give the desired compounds 12a-k.

N-(Benzo[d]thiazol-2-ylmethyl)pyrazine-2-carbamide (12a)

White micro crystals, m.p. 204–206 °C, yield 57% (0.25 g). IR: ν\textsubscript{max} \text{cm}^{-1} 3293, 3028, 2961, 1683, 1505, 1435, 899, 870, 770, 757. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ: 10.69 (s, 1H), 9.47 (s, 1H), 8.89–8.80 (m, 1H), 8.57 (d, J = 2.3 Hz, 1H), 8.44 (d, J = 9.1 Hz, 1H), 7.22 (t, J = 8.5 Hz, 1H), 6.97 (d, J = 7.5 Hz, 1H), 5.35 (d, J = 6.3 Hz, 2H). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) δ: 167.9, 163.5, 160.8, 152.8, 147.9, 147.6, 144.7, 144.6, 144.1, 143.0, 139.5, 137.0, 135.3, 132.1, 126.5, 125.6, 124.8, 123.1, 121.9, 120.5, 115.2, 41.8. HRMS m/z for C\textsubscript{13}H\textsubscript{12}N\textsubscript{3}OS (M + H\textsuperscript{+}) Calcd. 327.0764. Found: 327.0769.

(5)-N-(1-(Benzo[d]thiazol-2-yl)-2-methylpropyl) pyrazine-2-carboxamide (12b)

White micro crystals, m.p. 215–217 °C, yield 94% (0.41 g). IR: ν\textsubscript{max} \text{cm}^{-1} 3290, 3028, 2961, 1682, 1519, 1436, 1284, 899, 871, 740. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ: 9.37 (s, 1H), 8.76 (d, J = 7.7 Hz, 1H), 8.62–8.52 (m, 2H), 8.13 (s, 1H), 7.34 (d, J = 7.1 Hz, 1H), 1.76 (dd, J = 7.6, 1.2 Hz, 1H), 7.02 (t, J = 7.6 Hz, 1H), 6.83 (d, J = 8.7 Hz, 1H), 2.50–2.35 (m, 1H), 1.20 (d, J = 6.8 Hz, 3H), 1.05–1.00 (m, 3H). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) δ: 164.9, 162.4, 174.8, 147.6, 114.7, 143.9, 143.0, 142.8, 135.1, 128.1, 127.4, 124.5, 119.8, 116.6, 66.1, 34.4, 17.7, 17.4. HRMS m/z for C\textsubscript{13}H\textsubscript{12}N\textsubscript{3}OS (M + Na\textsuperscript{+}) Calcd. 327.3100. Found: 327.3101.
White microcrystals, m.p. 205–207°C, yield 25% (0.11 g). IR: ν_{max} cm⁻¹: 3368, 2931, 1672, 1518, 1443, 1274, 700, 796, 770, 766, 746, 2931, 1667, 1511, 1443, 1274, 700, 796, 770, 766, 746. H NMR (500 MHz, CDCl₃): δ: 9.39 (s, 1H), 9.18 (d, J = 8.5 Hz, 1H), 3.82 (d, J = 8.5 Hz, 1H), 2.00 (s, 3H), 1.27 (s, 6H). J = 8.5 Hz, 1H). 8.22 (d, J = 8.0 Hz, 1H), 7.70 (d, J = 8.0 Hz, 1H), 7.51 (t, J = 7.7 Hz, 1H), 5.55 (dd, J = 14.4, 5.9 Hz, 1H), 5.42 (dd, J = 14.4, 5.0 Hz, 2H), 3.50 (q, J = 7.0 Hz, 1H). 1H NMR (125 MHz, CDCl₃) δ: 167.8, 163.3, 153.0, 147.4, 147.5, 144.5, 144.0, 134.9, 133.5, 127.8, 126.5, 125.6, 124.2, 123.2, 121.8, 120.0, 109.4, 51.7, 50.7. HRMS m/z for C₂₈H₂₂N₂O₅ [M+H]⁺ Calcd. 480.5394. Found: 480.5399.

Preparation of pyrazinoic acid-benzothiazole conjugates 16

A dried heavy-walled Pyrex tube containing a small stir bar was charged with benzotriazole activated pyrazinoic acid 7 (0.7 mM) and 2-aminothiophenol 11 (0.7 mM) dissolved in THF (3 mL). The reaction mixture was exposed to microwave irradiation (50 W) at a temperature of 100°C for 2 h. Each mixture was allowed to cool through an inbuilt system until the temperature had fallen below 30°C (ca. 10 min). The reaction mixture was quenched with ice-cold water and the solid obtained was filtered and washed with Na₂CO₃ solution (10%) and water to give the desired compound 16.

2-(Pyrazin-2-yl)benzofuran (16)

White microcrystals, m.p. 201–203°C, yield 27% (0.12 g). IR: ν_{max} cm⁻¹: 3100, 2931, 1664, 1510, 1454, 978, 851, 767, 755, 736. H NMR (500 MHz, CDCl₃): δ: 9.67 (s, 1H), 8.70 (d, J = 10.3 Hz, 2H), 8.18 (d, J = 8.2 Hz, 1H), 8.01 (d, J = 8.0 Hz, 1H), 7.58 (t, J = 7.7 Hz, 1H), 7.50 (t, J = 7.2 Hz, 1H). 1C NMR (125 MHz, CDCl₃) δ: 160.8, 147.9, 147.6, 144.5, 143.9, 142.8, 136.0, 129.5, 124.8, 123.5, 121.3, 121.8, 53.0, 41.4. HRMS m/z for C₂₈H₂₂N₂O₅ [M-H]⁻ Calcd. 360.4350. Found: 360.4358.

Biological studies

MTT cytotoxicity assay

To assess the half-maximal cytotoxic concentration (CC₅₀), stock solutions of the tested compounds were prepared in 10% DMSO in ddH₂O and diluted further to the working solutions with DMEM. The cytotoxic activity of the compounds was tested in VERO-E6 cells by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with minor modifications. Briefly, the cells were seeded in 96 well-plates 100 μl/well at a density of 3 x 10⁴ cells/ml and incubated for 24 h at 37°C in 5% CO₂. After 24 h, cells were treated with various concentrations of the tested compounds in triplicates. 24 h later, the supernatant was discarded and cell monolayers were washed with sterile 1X phosphate buffer saline (PBS) 3 times, and MTT solution (20 μl of 5 mg/ml) was added to each well and incubated at 37°C for 4 h followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 μl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCl in 50 ml isopropanol). The absorbance of formazan solutions was measured at λ₅₇₈ nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.
The absorbance of cells without treatment – the absorbance of cells with treatment \times 100

\% \text{ Cytotoxicity} = \frac{\text{absorbance of cells without treatment}}{\text{absorbance of cells with treatment}} \times 100

The plot of \% cytotxicity versus sample concentration was used to calculate the concentration which exhibited 50 \% cytotocicity (IC_{50}).

I_{\text{CC}} \text{ determination}

In 96-well tissue culture plates, 2.4 \times 10^5 VERO-E6 cells were distributed in each well and incubated overnight at a humidified 37°C incubator under 5 \% CO_2 condition. The cell monolayers were then washed with 1× PBS and subjected to virus absorption (hCoV-19/Egypt/NRC-03/2020, Accession Number on GISAID: EPI_ISL_430820) for 1 h at room temperature (RT). The cell monolayers were further overlaid with 50 \mu l of DMEM containing varying concentrations of the test sample, followed by incubation at 37°C in 5 \% CO_2 incubator for 72 h. The cells were fixed with 100 \mu l of 4 \% paraformaldehyde for 20 min. and stained with 0.1 \% crystal violet in distilled water for 15 min. at room temperature. The crystal violet dye was then dissolved using 100 \mu l absolute methanol per well and the optical density of the color is measured at 570 nm using Anthos Zenyth 200 rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The IC_{50} of the compound is that required to reduce the virus-induced cytopathic effect (CPE) by 50 \%, relative to the virus control.[26–28,38]

QSAR studies

The synthesized agents revealing variable antiviral properties along with Favipiravir (standard reference) were utilized for developing the 2D-QSAR modeling by CODESSA-Pro (comprehensive descriptors for structural and statistical analysis) software. Geometry of the compounds was initially optimized by AM1 technique using hyperChem 8.0 and then uploaded to CODESSA-Pro for final geometrical structure optimization by MOPAC.[8,39] CODESSA-Pro calculated 808 molecular descriptors (constitutional, topological, geometrical, charge-related, semi-empirical, thermodynamic, molecular-type, atomic-type and bond-type descriptors) for the exported agents. Mathematical transformation of the experimental values (including IC_{50}, 1/logIC_{50} and 1/logIC_{50} mm) were used searching for the best QSAR model. The best multi-linear regression (BMLR) technique was utilized which is a stepwise search for the best n-parameter regression equations (where n stands for the number of descriptors used), based on the highest R^2 (square correlation coefficient), R^2cvOO (squared cross-validation "leave one-out, LOO" coefficient), R^2cVOO (squared cross-validation "leave many-out up to 20\% of the training set, LMO" coefficient), F (Fisher statistical significance criteria) values, and s^2 (standard deviation). The QSAR up to 3-descriptor model describing the biological activity of the antiviral active agents were generated (obeying the thumb rule of 5:7:1 which is the ratio between the data points and the number of QSAR descriptors).

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Conflict of Interest

The authors declare no conflict of interest

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