Syntheses of Isatins and Oxindoles Derivatives as SARS-CoV-2 Inhibitors Evaluated through Phenotypic Screening with Vero Cells

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To expand the variety of density functionalized compounds evaluated against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), we decided to prepare new acetylated and disubstituted 3-hydroxy bis-oxindoles and isoindigos coupling compounds using known protocols. The corresponding isatin derivatives were synthesized by ZrCl\textsubscript{4}/EtOH/reflux or HCl/AcOH/reflux coupling conditions using oxindole and functionalized isatins, furnishing new 3-hydroxy bis-oxindoles, which were dehydrated into new isoindigos. A total of 27 compounds bearing halogen, nitro and/or hydroxy groups on the isatin moiety, at the 3-, 5- and 7-positions, were prepared, including 5 new 3-hydroxy bis-oxindoles and 3 new halogenated isoindigos prepared according adapted procedures described in the literature. This library of nitrogen-isatin derivatives was evaluated against SARS-CoV-2 using a phenotypic screening assay. In this investigation, isatin derivatives 3d, 3e, 3h and 3i showed antiviral activity when tested at a single concentration. Compound 3e showed antiviral activity against SARS-CoV-2 in the concentration-response assay, however, it showed cellular toxicity in Vero cells. The present study identified substituted isatins as a promising new starting point for the development of anti-SARS-CoV-2 agents.

Keywords: 3-hydroxy bis-oxindoles, isoindigos, isatins, SARS-CoV-2, phenotypic VERO essay

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for the transmission and symptoms of the COVID-19 (coronavirus disease 2019). It was first reported in Wuhan Province, China, in December 2019.1 The infection has since spread worldwide, reaching as of September 2022, 608,000,000 cases and over 6,500,000 deaths worldwide.2 SARS-CoV-2 has been documented as the most infectious, fatal and pathogenic coronavirus after SARS-CoV (2002) and Middle East Respiratory Syndrome coronavirus (MERS-CoV, 2012) (SARS-CoV (800 deaths) and MERS-CoV (858 deaths)).3 From the above data it is clear that SARS-CoV-2 is much more dangerous and virulent as compared to other coronaviruses. SARS-CoV-2 is a positive-sense single-stranded RNA virus (+ssRNA).4 The similar names of SARS-CoV and SARS-CoV-2 were introduced by the scientific community due to the genome sequence similarity
of both coronaviruses, which could vary from 86%5 until 89.1%.1

To date, some drugs have shown to have activity against SARS-CoV-2 in vitro and in vivo, with remdesivir6 being the first small-molecule antiviral approved for COVID treatment by the US Food and Drugs Administration (FDA). Yet, clinical effects and efficacy of the drug remdesivir are still controversial.6,7,8 Despite the development of successful new antivirals and vaccines for COVID, the development of new drugs for SARS-CoV-2 treatment continues. Several efforts are focused on the development of molecules that can inhibit the essential major polyprotein processing, 3CLpro (Type 3 chymotrypsin protease),10 which is crucial for the viral replication.11

After the transcription of its genome, the betacoronavirus genus produces a polypeptide of approximately 800 kDa, which is proteolytically cleaved to generate several proteins. This proteolytic processing is mediated by proteases such as PLpro (Papain-like protease) and 3CLpro. 3CLpro is the main protease found in the coronavirus. It consists of a 33.8 kDa cysteine protease which mediates the maturation of functional polyproteins involved in the assembly of the transcriptional machinery of viral replication. 3CLpro cleaves the polyprotein at 11 conserved sites, generating non-structural proteins that play an important role in viral replication. Furthermore, 3CLpro is located at the 3’ end, exhibiting high variability levels.12,13

3CLpro plays an important role in polyproteins hydrolysis generating non-structural proteins (NSPs). Therefore, inhibitors of these proteases can block the generation of non-structural proteins. This enzyme is necessary for the proteolytic maturation of viral polyproteins (pp1a and pp1ab) to form the RNA replicase-transcriptase complex, which is essential for both viral transcription and replication processes.15,16 These proteases are essential tools for the self-cleavage process and play a key role in viral particle replication and infection, which makes it recognized as a high potential target for drugs that aim to control SARS-CoV-2 infection.17 Now if we can covalent binding small molecules to this nucleophilic center, this will lead to the deactivation of 3CLpro, inhibiting its bind to ACE2 (angiotensin-converting-enzyme) receptor of the human body causing no infection by SARS-CoV-2.18

ACE2 recognition by the S protein enables SARS-CoV-2 invasion. Therefore, the decrease in the activity and expression of ACE2 in the membrane of the cell reduces the entry capacity and inflammatory activity of the virus.19 Considering this information, the ideal compound would be one that has the ability of destabilizing the interaction between S protein and ACE2 receptor, causing the inhibition of virus entry as well as interrupting the activity of enzymes involved in SARS-CoV-2 replication cycle.

Several small molecules screened from various chemical libraries have been identified as potent SARS-CoV-2 protease inhibitors.20 However, some of these small molecules may be unsuitable for easy structural modification. Consequently, pharmacophoric scaffolds, which are easy to synthesize and chemically modify must be explored. In this direction, isatin derivatives occupy a prominent position as synthetic platform for drug design to an antiviral candidate.21 For example, N-alkylated isatin derivatives 1a and 1b showed high half-maximal inhibitory concentration (IC50) values against SARS-CoV,22 which resembles to the SARS-CoV-2 with 86% of similarity (Figure 1). Computational modeling revealed that steric effects from N-alkylated side chain was crucial for inhibitory potency, as well as the hydrophobicity and presence of electron withdrawing groups such as bromo and nitro on the isatin core. Zhou et al.23 also reported isatin derivatives substituted at the N-1 and C-5 positions. In their studies, the N-alkylated C-5 substituted carboxamide group 2 was the most active compound against SARS-CoV 3C-like protease. According to the authors, the carboxamide assists the occupation of the active site through hydrogen bonding interactions. Due this prominent position of isatin derivatives by its easily chemical modifications and antiviral activities, we decided to synthesize new 3-hydroxy bis-oxindoles, isoindigos and evaluated them, together with the isatins used in their preparation, against SARS-CoV-2 coronaviruses.

![Figure 1: Structures of isatin derivatives with inhibitory activity against SARS-CoV.](image)

**Experimental**

**Chemistry**

**Materials and methods**

Solvents and reagents were purchased from commercial suppliers (Sigma-Aldrich, Darmstadt, Germany; Alfa Aesar, Kandel, Germany; ABCR ACROS Organics, Schwerte, Germany and Fischer Scientific, Schwerte, Germany). Nuclear magnetic resonance (NMR) spectra were recorded using the following spectrometers from Bruker (Ettlingen, Germany) in deuterated solvents from Deutero and
H NMR data are reported as follows: chemical shifts were referenced to the residual solvent signal (CDCl₃: δH = 7.26 ppm, δC = 77.16 ppm; CD3OD: δH = 2.50 ppm, δC = 39.52 ppm; CD3N: δH = 8.74 ppm, δC = 150.35 ppm). The evaluation and assignment of the spectra was achieved using the software MestReNova from Mestrelab Research. 1H NMR data are reported as follows: chemical shift (parts per million, ppm), multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet), coupling constant (J in Hz), integration and assignment. High-performance liquid chromatography (HPLC) analysis was recorded on HPLC-UV system Shimadzu (Kyoto, Japan) equipped with binary pump DGU-20A5 Prominence, with a diode array detector (DAD) SPD-M20A. Electrospray ionization high resolution mass spectrometry (ESI-HRMS) spectra were recorded on Waters Q-TOF- Ultima III instrument from Waters (Milford, USA) with a dual source and a suitable external calibrant. IR spectra were recorded on routine Fourier transform infrared (FTIR) spectrometer (Bruker Optics Tensor 27, Ettlingen, Germany) using a diamond ATR unit. The evaluation of the spectra was achieved using the software Opus 6.5 from Bruker. FTIR device from Shimadzu IRAffinity-1 (Kyoto, Japan) with compressed tablets of anhydrous potassium bromide and FTIR-ATR Optics Tensor 27, Ettlingen, Germany) using a diamond crystal cell on the equipment model Cary 630 Agilent equipped with attenuated total reflectance (ATR) with diamond crystal cell and deuterated triglycine sulfate detector (DTGS). Melting point ranges were determined with I9000 series digital melting point apparatus from ThermoFischer (Waltham, USA) and MQAPF-302 instrument (Cotia, Brazil) and were uncorrected.

Synthesis of isatin and acetylated isatin derivatives

Compounds 5-bromo-1H-indole-2,3-dione (3b), 5-chloro-1H-indole-2,3-dione (3c), 5-nitro-1H-indol-2,3-dione (3d), 5,7-dibromo-1H-indole-2,3-dione (3e), 5,7-dichloro-1H-indole-2,3-dione (3f), 5-chloro-7-bromo-1H-indole-2,3-dione (3g), N-acetylindoline-2,3-dione (3h), N-acetyl-5-bromoindoline-2,3-dione (3i), N-acetyl-5-chloroindoline-2,3-dione (3j), N-acetyl-5-nitroindoline-2,3-dione (3k), 3-hydroxy-(3,3'-biindoline)-2,2'-dione (3l), 5-bromo-3-hydroxy-(3,3'-biindoline)-2,2'-dione (3m), 5-chloro-3-hydroxy-(3,3'-biindoline)-2,2'-dione (3n), and 3-hydroxy-5-nitro-(3,3'-biindoline)-2,2'-dione (3o) were synthesized as described in the literature. For more details, see Supplementary Information (SI) section.

General procedures for synthesis of 3-hydroxy bis-oxindoles

(E)-(3,3'-Biindolylidene)-2,2'-dione (5a), (E)-5-bromo-(3,3'-biindolylidene)-2,2'-dione (5b), (E)-5-chloro-(3,3'-biindolylidene)-2,2'-dione (5c), and (E)-5-nitro-(3,3'-biindolylidene)-2,2'-dione (5d) were synthesized as described in the literature. For more details, see SI section.

(i) Method A: 24 indole-2,3-dione derivatives (3a-3g, 3i-3j, 0.5 mmol), indolin-2-one (6, 0.5 mmol) and ZrCl₄ (23 mg, 0.1 mmol) were heated in anhydrous ethanol (4 mL) under reflux overnight. The mixture was slowly cooled to room temperature. The colored solids precipitated and were collected by filtration, then washed by a small amount of anhydrous ethanol to deliver dehydrated product 3-hydroxy-3,3'-biindoline-2,2'-dione (4a-4i). The obtained compounds were dried in the oven.

(ii) Method B: 25 indolin-2-one (6, 0.5 mmol) and substituted indole-2,3-dione (3a-3g, 3i-3j, 0.5 mmol) were dissolved in glacial acetic acid and one drop of concentrated hydrochloric acid was added. The solution was stirred under reflux overnight. The resultant mixture was concentrated under vacuum. The colored solids precipitated and were collected by filtration, then washed by a small amount of anhydrous ethanol to deliver dehydrated product 3-hydroxy-3,3'-biindoline-2,2'-dione (4a-4i). The obtained compounds were dried usually as a purple red powder.

5,7-Dibromo-3-hydroxy-(3,3'-biindoline)-2,2'-dione (4e)

Purple solid; mp > 390 ºC; yield 73%; IR (ATR) ν/cm⁻¹: 3145, 1703, 1609, 1555, 1445, 1335, 1309, 1156, 865, 604; 1H NMR (400 MHz, DMSO-d₆) δ 10.91 (s, 1H), 10.28 (s, 1H), 7.61 (d, 1H, J 1.8 Hz), 7.57 (d, 1H, J 7.3 Hz), 7.31 (t, 1H, J 7.6 Hz), 7.06 (t, 1H, J 7.5 Hz), 6.92 (s, 1H), 6.80 (d, 1H, J 7.7 Hz), 6.04 (s, 1H), 4.03 (s, 1H); 13C NMR (100 MHz, DMSO-d₆) δ 176.2, 173.7, 143.4, 141.7, 134.2, 131.8, 128.9, 126.7, 125.6, 125.1, 121.4, 113.0, 109.2, 102.9, 76.2, 51.6; HRMS (APCI) m/z, calcd. for C₁₈H₂₃BrNO₂ [M – H₂O]: 417.8953, found: 417.8951.

5,7-Dichloro-3-hydroxy-(3,3'-biindoline)-2,2'-dione (4f)

Purple solid; mp > 390 ºC; yield 61%; IR (ATR) ν/cm⁻¹: 3202, 1730, 1693, 1619, 1454, 1152, 742; 1H NMR (400 MHz, DMSO-d₆) δ 11.04 (s, 1H), 10.27 (s, 1H), 7.58 (d, 1H, J 7.3 Hz), 7.42 (d, 1H, J 2.0 Hz), 7.32 (t, 1H, J 7.8 Hz), 7.06 (t, 1H, J 7.2 Hz), 6.93 (s, 1H), 6.81 (d, 1H, J 7.7 Hz), 5.91 (d, 1H, J 1.6 Hz), 4.05 (s, 1H); 13C NMR (100 MHz, DMSO-d₆) δ 176.9, 173.9, 143.6, 139.9, 131.7, 129.2, 129.1, 126.9, 125.6, 125.3, 122.6, 121.6, 114.7, 109.4, 76.2, 51.7; HRMS (APCI) m/z, calcd. for C₁₈H₂₃Cl₂NO₂ [M – H₂O]: 329.9963, found: 329.9959.
7-Bromo-5-chloro-3-hydroxy-(3,3′-biindoline)-2,2′-dione (4g)

Red solid; mp > 390 °C; yield 63%; IR (ATR) v/cm⁻¹ 3144, 3068, 1703, 1614, 1451, 1335, 1307, 868, 746; ¹H NMR (400 MHz, DMSO-d₆) δ 10.91 (s, 1H), 10.28 (s, 1H), 7.57 (d, 1H, J 7.3 Hz), 7.50 (d, 1H, J 2.0 Hz), 7.31 (t, 1H, J 7.7 Hz), 7.05 (t, 1H, J 7.5 Hz), 6.93 (s, 1H), 6.80 (d, 1H, J 7.7 Hz), 5.92 (s, 1H), 4.03 (s, 1H); ¹³C NMR (100 MHz, DMSO-d₆) δ 176.3, 173.3, 143.0, 141.0, 131.3, 131.0, 128.5, 126.3, 125.2, 124.7, 122.3, 121.0, 108.8, 102.1, 75.8, 51.1; HRMS (APCI) m/z, calced for C₁₆H₈BrClN₂O₂: 373.9458, found: 373.9456.

N-Acetyl-5-bromo-3-hydroxy-(3,3′-biindoline)-2,2′-dione (4i)

Brown solid; mp > 390 °C; yield 61%; IR (ATR) v/cm⁻¹ 3345, 1746, 1714, 1620, 1470, 1299, 1188, 1164, 832; ¹H NMR (300 MHz, DMSO-d₆) δ 10.32 (s, 1H), 8.01 (d, 1H, J 8.7 Hz), 7.67 (d, 1H, J 7.3 Hz), 7.52 (dd, 1H, J 8.7, 2.2 Hz), 7.52 (dd, 1H, J 8.7, 2.2 Hz), 7.35 (t, 1H, J 7.7 Hz), 7.23 (s, 1H), 7.12 (t, 1H, J 7.2 Hz), 6.80 (d, 1H, J 7.7 Hz), 6.25 (d, 1H, J 2.2 Hz), 4.20 (s, 1H), 2.64 (s, 3H); ¹³C NMR (75 MHz, DMSO-d₆) δ 177.3, 174.2, 170.7, 144.0, 139.6, 133.3, 130.2, 129.7, 127.1, 126.9, 125.2, 122.2, 118.3, 117.1, 110.0, 75.6, 53.8, 26.6; HRMS (ESI) m/z, calced for C₁₆H₁₄BrClN₄O₂: 400.0059, found: 400.0057.

N-Acetyl-5-chloro-3-hydroxy-(3,3′-biindoline)-2,2′-dione (4j)

Rose solid; mp > 390 °C; yield 56%; IR (ATR) v/cm⁻¹ 3338, 1743, 1712, 1617, 1403, 1183, 1076, 1030, 834; ¹H NMR (300 MHz, DMSO-d₆) δ 10.30 (s, 1H), 8.06 (d, 1H, J 8.8 Hz), 7.66 (d, 1H, J 7.4 Hz), 7.44-7.27 (m, 2H), 7.22 (s, 1H), 7.15-7.03 (m, 1H), 6.79 (d, 1H, J 7.7 Hz), 6.10 (d, 1H, J 2.3 Hz), 4.20 (s, 1H), 2.63 (s, 3H); ¹³C NMR (75 MHz, DMSO-d₆) δ 177.3, 174.1, 170.5, 143.8, 139.1, 130.3, 129.8, 129.6, 127.0, 125.1, 123.8, 122.1, 117.8, 109.9, 75.5, 53.6, 26.4; HRMS (ESI) m/z, calced for C₁₆H₁₄ClN₄O₂: 399.0984, found: 399.0982.

General procedure for synthesis of isodindigos

Undehydrated 3-hydroxy bis-oxindoles (0.2 mmol) from general procedures A or B were placed in an inert atmosphere under reflux overnight in dry toluene (2 mL) in acidic medium (one drop of sulfuric acid). The crude was filtered, washed with ethyl acetate (2 × 2 mL) and dried under vacuum to furnish the corresponding isodindigos as colored solids.

(È)-5,7-Dibromo-(3,3′-biindolylidene)-2,2′-dione (5e)

Purple solid; mp > 390 °C; yield 45%; IR (ATR) v/cm⁻¹ 3170, 1609, 1444, 1307, 1151, 1017, 864; ¹H NMR (400 MHz, DMSO-d₆) δ 11.32 (s, 1H), 11.04 (s, 1H), 9.33 (t, 1H, J 5.6 Hz), 9.03 (d, 1H, J 7.8 Hz), 7.82 (s, 1H), 7.39 (t, 1H, J 7.6 Hz), 7.00 (t, 1H, J 7.8 Hz), 6.84 (d, 1H, J 7.9 Hz); HRMS (APCI) m/z, calced for C₁₆H₁₂Br₃N₄O₂: 417.8953, found: 317.8944.

(Vero CCL-81 (ATTC) cells were cultured in high glucose DMEM medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, Waltham, USA) and 100 U mL⁻¹ of penicillin and 100 μg mL⁻¹ Streptomycin (Thermo Scientific) at 37 °C with 5% CO₂.

Virus strain

All procedures involving the SARS-CoV-2 virus were performed in the level 3 biosafety laboratory of the Institute of Biomedical Sciences of the University of São Paulo. The SARS-CoV-2 virus used in this study (H1AE-02: SARS-CoV-2/SP02/human/2020/ARB, GenBank Accession No. MT126808.1) was isolated from a nasopharyngeal sample of a confirmed COVID-19 patient at Hospital Israelita Albert Einstein, São Paulo (SP) Brazil.26

Phenotypic screening with SARS-CoV-2

For phenotypic screening of compounds, 2000 Vero CCL-81 (ATCC) cells were seeded per well in 384-well plates in high glucose Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo
Cells were incubated for 24 h at 37 °C with 5% CO₂. Compounds were diluted to 20 mM in dimethylsulfoxide (DMSO).

Before performing cell treatment, compounds were diluted 33.33× in phosphate-buffered saline (PBS), and 10 μL from each well was transferred to assay plates, thus having a final dilution factor of 200×. Initially the compounds were tested at a single concentration of 10 μM. Chloroquine (Sigma-Aldrich) was used as viral inhibition control in concentration-response curves, starting at 50 μM, with 10-concentration points and 2-fold dilutions. Compounds were also tested on a dose-response curve in which they were serially diluted and manually transferred to a 384-well polypropylene plate (Greiner Bio-One, Frickenhausen, Germany) containing sterile phosphate-buffered saline (PBS) pH 7.4, for a final dilution factor of 33.3. Then, 10 μL from each well in the compound plate was transferred to the assay plate containing cells, followed by the addition of SARS-CoV-2 viral particles to the cells at 0.1 multiplicity of infection (MOI) in 10 μL DMEM high glucose per well. After viral adsorption, high glucose DMEM medium supplemented with 6% fetal bovine serum was added. After 33 h of incubation at 37 °C, 5% CO₂, the plate was fixed in 4% paraformaldehyde in PBS pH 7.4 and subjected to indirect immunofluorescence detection of viral cell infection. Three independent trials were performed.

For the detection of SARS-CoV-2 viral infection, the hyperimmune serum of a COVID-19 convalescent patient diluted 1:1000 in 5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) in PBS (v/v) was used as the primary antibody. After incubation, the wells were washed and a solution containing Alexa488-conjugated goat anti-human IgG (Thermo Fisher Scientific, Waltham, MA, USA) and a solution containing Alexa488-conjugated goat anti-human IgG (Thermo Fisher Scientific, Waltham, MA, USA) was added. After 33 h of incubation at 37 °C, 5% CO₂, the plate was fixed in 4% paraformaldehyde in PBS pH 7.4 and subjected to indirect immunofluorescence detection of viral cell infection. Three independent trials were performed.

Image acquisition was performed on the Operetta High Content Imaging System (PerkinElmer, Waltham, MA, USA) using a 20× magnification objective and image analysis was performed using Harmony software (PerkinElmer), version 3.5.2. The analysis consisted of identification and counting of Vero CCL-81 cells based on nuclear targeting and viral infection based on cytoplasmic staining detected by the immunofluorescence assay. The infection rate (IR) was calculated as the ratio of the number of infected cells to the number of total cells counted in each well. Cell survival rate was calculated as the number of cells counted in each well divided by the average number of cells in the positive control wells (DMSO-treated infected cells), multiplied by 100. Antiviral activity was determined by normalizing the IR to the control negative (infected and uninfected cells treated with DMSO) as described. Concentration response curves were plotted using normalized activity and cell survival at each concentration. These two parameters were used to calculate EC₅₀ and CC₅₀ concentration, concentrations of compounds that reduce infection rate and cell survival by 50%, respectively, compared to untreated infected controls of each compound using GraphPad Prism version 9.0.29

### Results and Discussion

### Chemistry

The synthesized compounds were divided into three classes based on their structural complexity. Class 1 comprised isatins 3a-3k with different substituents on the aromatic ring in the C-5 and/or C-7 position, as well as a N-acetyl protecting group. In this class we observed no N-acetylation (Ac₂O, reflux) when a C-7 substituent (Br, Cl or NO₂) was present. Class 2 consisted of 3-hydroxy bis-oxindoles 4a-4g, 4i and 4j, which may be viewed as a 3-hydroxylated two 3,3'-linked oxindoles. Finally, class 3 (isoindigos 5a-5g) were compounds structurally similar to class 2, except by an 3,3'-exocyclic double bond joined both oxindole structures. The structures of class 1-3 compounds are given in Figure 2. Of the 27 compounds prepared to this investigation, 19 members have been reported in the literature (see SI section for more details).

Our synthetic strategy to synthesize new disubstituted 3-hydroxy bis-oxindoles 4e-4g and isoindigos 5e-5g was based on known coupling methodologies described in the literature. In this direction, oxindoles 6 was reacted with isatins 4e-4g using ZrCl₄ in refluxing ethanol (method A) or HCl in refluxing acetic acid (method B, Scheme 1). However, under both conditions no syntheses of isoindigos 5e-5g were accomplished, and only the new intermediates 3-hydroxy bis-oxindoles 4e-4g were obtained (Table 1). We reasoned those results due strong withdrawing effect of two halogen substituents on isatin moiety, which would make coordination of the hydroxyl group less effective and dehydration more difficult. The 3-hydroxy intermediates obtained were easily identified by NMR analysis: HO–C₃–H was displayed at δ 4.03-4.05 ppm for 4e-4g and at δ 4.19-4.22 ppm for acetylated 4h and 4i, while hydroxylated C₃ was displayed ranging δ 76.2-75.6 ppm. We also observed during our synthetic efforts that N-acetyl protecting group on isatin partner were normally removed under coupling conditions, except when monohalogenated isatins 3i and 3j were coupled employing the method B, leading to new 4i and 4j compounds, respectively. Finally, 3-hydroxy bis-oxindoles 4e-4g were dehydrated under more
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acidic conditions to furnished desired new isoindigos 5e-5g, respectively, in moderate yields.

Biological evaluation

Phenotypic assay with SARS-CoV-2

A high content screening assay (HCS) was designed to evaluate compounds that inhibit infection and cytoxicity in Vero cells infected with a SARS-CoV-2 isolate. The potential antiviral activity of 27 compounds against SARS-CoV-2 in Vero CCL-81 cells was evaluated. For this, an initial screening was performed, and the compounds were tested at a single concentration of 10 μM, as shown in Table 2.

Table 1. New 3-hydroxy bis-oxindoles 4e-4g, 4i and 4j synthesized

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</tr>
<tr>
<td>3n</td>
<td>110.64</td>
<td>40.08</td>
</tr>
<tr>
<td>3o</td>
<td>102.95</td>
<td>47.53</td>
</tr>
<tr>
<td>3p</td>
<td>97.15</td>
<td>22.47</td>
</tr>
<tr>
<td>3q</td>
<td>104.47</td>
<td>6.96</td>
</tr>
<tr>
<td>3r</td>
<td>101.33</td>
<td>2.35</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>121.81</td>
<td>96.96</td>
</tr>
</tbody>
</table>

Figure 2. Compounds synthesized and evaluated against SARS-CoV-2.

Scheme 1. Synthetic pathway to new 3-hydroxy bis-oxindoles: (a) ZrCl4, EtOH, reflux, overnight, (b) HCl conc., AcOH, reflux, overnight; and their dehydration to new isoindigos.

Table 2. Anti SARS-CoV-2 activity by Vero CCL-81 cells-high content screening-at 10 μM (CS: cell survival; AA: antiviral activity)

<table>
<thead>
<tr>
<th>Compound</th>
<th>CS %</th>
<th>AA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a (isatin)</td>
<td>109.38</td>
<td>25.05</td>
</tr>
<tr>
<td>3b</td>
<td>169.37</td>
<td>45.11</td>
</tr>
<tr>
<td>3c</td>
<td>51.15</td>
<td>30.46</td>
</tr>
<tr>
<td>3d</td>
<td>97.10</td>
<td>66.30</td>
</tr>
<tr>
<td>3e</td>
<td>79.54</td>
<td>64.92</td>
</tr>
<tr>
<td>3f</td>
<td>75.16</td>
<td>31.78</td>
</tr>
<tr>
<td>3g</td>
<td>95.26</td>
<td>35.49</td>
</tr>
<tr>
<td>3h</td>
<td>64.19</td>
<td>76.28</td>
</tr>
<tr>
<td>3i</td>
<td>91.39</td>
<td>61.48</td>
</tr>
<tr>
<td>3j</td>
<td>98.85</td>
<td>61.48</td>
</tr>
<tr>
<td>3k</td>
<td>76.97</td>
<td>33.56</td>
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<tr>
<td>3l</td>
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<td>121.81</td>
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</tr>
</tbody>
</table>
Compounds that show antiviral activity greater than 60% and cell survival greater than 75% in the primary screening at a concentration of 10 μM (3d, 3e, 3h and 3i, Figure 3) were tested in dose-response, with an initial concentration of 100 μM (Figure 4). These compounds belong to class 1 of isatins, with different substituents on the aromatic ring at the C5 and/or C7 position and/or acetylation of nitrogen. Compound 3e showed activity against SARS-CoV-2 in the dose-response assay, however it showed cellular toxicity in Vero cells (Table 3).

**Table 3. Inhibitory activities of compounds tested in concentration-response**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC_{50}/μM</th>
<th>CC_{50}/μM</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3d</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1</td>
</tr>
<tr>
<td>3e</td>
<td>3.6</td>
<td>4.1</td>
<td>1.1</td>
</tr>
<tr>
<td>3h</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1</td>
</tr>
<tr>
<td>3i</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1</td>
</tr>
</tbody>
</table>

EC_{50}: half maximum effective concentration; CC_{50}: half maximum cytotoxic concentration; SI: selectivity index (CC_{50}/EC_{50}).

**Figure 3.** Compounds with antiviral activity in the primary screening against SARS-CoV-2.

**Figure 4.** SARS-CoV-2 antiviral concentration-response curves for selected compounds. Chloroquine (CQ) was used as a positive control for viral infection inhibition. Represented *in vitro* antiviral activity (blue curves) and cytotoxic activity against Vero cells (red curves) against SARS-CoV-2. Three independent experiments were carried out.
Conclusions

This investigation allowed the synthesis of 8 new isatin derivatives (5 new 3-hydroxy bis-oxindoles 4e-4g, 4i and 4j and 3 new isoindigos 5e-5g) in moderate yields, contributing to the expansion of this important derivative synthesis strategy. In addition, another 19 known compounds were prepared and all compounds were evaluated for their SARS-CoV-2 count properties through tests employing Vero cells.

Unfortunately, all the new compounds were found to be inactive against COVID-19 disease by the Vero cells evaluation. Among the other compounds evaluated, the brominated isatin derivatives (3d and 3i), another which contain the nitro group (3d) and the N-acetylated isatin 3h, showed the best antiviral activities by initial screening. Among active isatins, compound 3e showed activity against SARS-CoV-2 in the dose-response assay, however it showed cellular toxicity in Vero cells. An important observation regarding structure-activity seems to be simplicity: while the 5,7-dibrominated 3e and 5-nitro 3d isatins showed antiviral activity, the respective 3-3' conjugated brominated derivatives 4e and 5e and with nitro group 4d and 5d showed lower activities. These results are in agreement with previous results described in the literature, where isatins showed previously promise of antiviral activity as an inhibitor of SARS-CoV-2 main protease (3CL\textsuperscript{pro} or M\textsuperscript{pro}), both \textit{in silico} and \textit{in vitro}.\textsuperscript{30-32} However, despite the 3-hydroxy 5,7-dibrominated bisoxindole derivative 4e showing high antiviral activity, it showed high cellular toxicity. The promising results of antiviral activity obtained with the simplest isatin derivatives 3d, 3e, 3h and 3i show that more detailed investigations need to be carried out.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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Author Contributions

Cintia M. C. F. Lima was responsible for organic synthesis work; Lácio H. Freitas-Junior for coordination of biological assays work; Carolina B. Moraes for biological assays work; Cecília G. Barbosa for biological assays work, contributions to manuscript writing; Till Opatz for coordination of organic synthesis work, contributions to manuscript writing; Mauricio M. Victor for coordination of organic synthesis work, contributions to manuscript writing.

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