Microwave-assisted synthesis, antimicrobial, antiquorum-sensing and cytotoxic activities of a new series of isatin-β-thiocarbohydrazones

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\textit{In silico} studies

\textit{In silico} methods are fast computer studies that are useful in the evaluation of physicochemical properties, pharmacokinetcis and toxicity of compounds.\textsuperscript{[1]} Lipophilicity and aqueous solubility are the principal properties that affect drug absorption. Thus, the new analogs were studied for the prognosis of Lipinski’s rule of five\textsuperscript{[2]} and Veber’s criteria.\textsuperscript{[3]}

\textit{Molinspiration} calculations

Lipinski’s rule of five is valuable in the prognosis of oral absorption of drugs\textsuperscript{[2]} and it is relied on the physicochemical properties of the investigated compounds. Also, topological polar surface area (TPSA) and number of rotatable bonds (Nrotb) affect drug absorption.\textsuperscript{[3]} The calculated miLogP values for the investigated compounds (except 15-17) were < 5, which is the upper limit for the drugs to be able to penetrate through biomembranes according to Lipinski’s rule. Thus, these compounds are expected to exhibit good oral bioavailability (Supplementary Table S1). All tested compounds (except 5) have TPSA values < 140 Å\textsuperscript{2}; thus, they are expected to have acceptable intestinal absorption (Supplementary Table S1). It is worth mentioning that all compounds have zero or one violation of Lipinski’s rule; therefore, they are expected not to have problems in bioavailability (Supplementary Table S1). Two or more violations suggest the probability of problems in bioavailability.
Experimental

Chemistry

Synthesis of 3-amino-1-[(5-methyl-2-oxo-2,3-dihydro-1H-indol-3-ylidene)amino]thiourea (3)

A mixture of thiocarbohydrazide (1) (1.06 g, 0.01 mol), 5-methylisatin (2) (1.612 g, 0.01 mol) and glacial acetic acid (0.1 mL) in ethanol (10 mL) was heated under microwave irradiation (50 W) at 100 °C for 15 min. The precipitated solid upon cooling was filtered, dried and crystallized from ethanol to give compound 3.

Synthesis of hybrid compounds 4-17

A mixture of 3 (0.249 g, 0.001 mol), aldehyde (0.001 mol) and glacial acetic acid (0.1 mL) in ethanol (5 mL) was heated under microwave irradiation (50 W) at 100 °C for 15 min. The orange precipitate attained was filtered and crystallized from ethanol to yield 4-17.

Biological screening

Antimicrobial and antiquorum-sensing testing

Antibacterial testing

All the bacterial strains were propagated in Luria Bertani (LB) broth (1% peptone, 0.5% yeast extract, 0.5% NaCl) and solidified with 1.5% agar. Melted Muller Hinton agar (50 mL) at 50 °C were seeded with 50 µl of 1x10^6 CFU/mL of 18 hrs culture of the tested bacteria. The inoculated agar was mixed and poured into 15-cm-diameter plates to solidify. Wells were made in agar using cork borer. Tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL. Aliquots each of (100 µl) of each compound were applied into the wells. DMSO was also included as a negative control, and ampicillin in a concentration of 5 mg/mL was used as a reference antibacterial agent. The compounds were allowed to diffuse for 2 hrs at 4 °C and incubated at 37 °C for 24 hrs. Inhibition zones were measured using Vernier caliper and the activity of the tested compounds was estimated in comparison to ampicillin (Table 1). The inhibition zone diameter of DMSO was subtracted from the antibacterial activity of the tested compounds.

Antifungal testing

Determination of diameter of inhibition zone (mm)

Sabouraud medium (50 mL) inoculated with 50 µl of 1x10^6 CFU/mL of 24 hrs culture of Candida albicans. Glucose minimal medium (50 mL) was inoculated with 50 µl of 1x10^3 SFU/mL of Aspergillus fumigatus 293. Wells were made in agar using cork borer. The tested
compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL and 100 µl of test solution was applied in the wells. The standard antifungal drug (fluconazole) was also added at the same concentration to each plate. In addition, DMSO (control solvent) was added to each plate. Plates were incubated at 37 °C for 48 hrs.\textsuperscript{[6-9]} Antifungal activity of the tested compounds was determined by measuring the inhibition zone diameter (Table 1). The inhibition zone diameter of DMSO was subtracted from the antifungal activity of tested compounds.

\textit{Determination of minimal inhibitory concentrations (MICs)}

Minimal inhibitory concentrations (MICs) were determined by serial dilution technique using 96-multiwell microtiter plates. The investigated compounds 4-17 were dissolved in DMSO 100% to prepare stock solutions of 5000 µg/mL. Two fold serial dilutions of the dissolved compounds were performed in glucose minimal medium providing eight different concentrations (2500, 1250, 625, 312.5, 156.25, 78.125, 39.06 and 19.53 µg/mL). \textit{A. fumigatus} 293 was diluted to 1x10\textsuperscript{3} SFU/mL in glucose minimal medium, and \textit{C. albicans} diluted to 1x10\textsuperscript{6} CFU/mL in Sabouraud's medium. 20 µL of the diluted culture was added to the test solutions (50 µL) in the wells (one well per concentration). The plates were incubated at 37 °C for 48 hrs for \textit{C. albicans} and at 30 °C for 48 hrs for \textit{A. fumigatus} 293.\textsuperscript{[6,8,9]} MIC was detected visually as the least concentration inhibiting fungal growth (no turbidity) and the activity of the tested compounds was estimated in comparison to fluconazole (Table 2).

\textit{Antiquorum-sensing testing}

The culture was prepared by growing \textit{C. violaceum} ATCC 12472 in LB broth and incubated for 16-18 hours in an orbital incubator running at 28 °C and 150 rpm. The culture was then adjusted to 0.5 McFarland standard (Ca. 1x10\textsuperscript{6} CFU/mL). \textit{C. violaceum} (50 µL) was inoculated into LB agar (50 mL), poured into plates and solidified. Wells were made in LB agar medium using cork borer. The tested compounds, 4-17 were dissolved in DMSO 100% in eppendorff tubes for final concentration 5 mg/mL, and 50 µL of the test solution was applied into the wells. The positive control (catechin) was also added at the same concentration and volume to each plate. In addition, DMSO (control solvent) was added to each plate. Plates were incubated at 30 °C for 48 hours to check the inhibition of pigment production around the wells. Bacterial growth inhibition would result in a clear halo around the disc, while a positive quorum sensing inhibition is exhibited by a turbid halo harboring pigmentless bacterial cells of \textit{C. violaceum} ATCC 12472 monitor strain.\textsuperscript{[6,10]} Bacterial growth inhibition by the tested compounds was measured as radius (r\textsubscript{1}) in mm, while both growth and pigment inhibition was measured as radius (r\textsubscript{2}) in mm. The pigment inhibition (QS inhibition) was determined by subtracting bacterial growth inhibition (r\textsubscript{1}) from total radius (r\textsubscript{2}); thus, QS inhibition = (r\textsubscript{2}-r\textsubscript{1}) in mm (Table 1).
In vitro cytotoxicity testing

Compounds 4-17 were screened for cytotoxic activity against cervical (Hela) and kidney fibroblast (COS-7) cancer cell lines using MTT assay.\textsuperscript{11-13} Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) at 37 °C in an atmosphere of 5% CO\textsubscript{2} were added.

Cells were placed in a 96-multiwell microtiter plate (10\textsuperscript{4} cells/well), for 24 hrs at 37 °C and in an atmosphere of 5% CO\textsubscript{2} before treatment with the compounds to allow attachment of the cells to the wall of the plate. The tested compounds were dissolved in DMSO and diluted with phosphate buffer solution (PBS) to obtain different concentrations. Tested compounds of different concentrations were added to each well and cells were incubated with the compounds for 48 hrs at 37 °C and in an atmosphere of 5 % CO\textsubscript{2}. All tests were performed in triplicates using doxorubicin as a reference antitumor agent. The treated cells were washed with PBS and 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) (5 mg/mL MTT stock in PBS diluted to 1 mg/mL with 10% RPMI-1640 medium) was added. The 96-multiwell plates were read by microarray reader Perkinelmer vector 3V multilabel counter model 1420 (Perkinelmer, Boston, MA) for optical density at 490 nm.\textsuperscript{11-13} The relative percentage cell viability was calculated from the following equation:

\[
\% \text{ cell viability} = \frac{A_{\text{treated cells}} - A_{\text{blank}}}{A_{\text{untreated cells}} - A_{\text{blank}}} \times 100
\]

The relation between surviving fraction and drug concentration is plotted to get the survival curve for cervical cancer (Hela) and kidney fibroblast cancer (COS-7) cell lines. The concentration required for 50% inhibition of cell viability (IC\textsubscript{50}) was obtained for each compound from the curve fitting using Sigma plot10.
Supplementary Table S1. TPSA, Nrotb and calculated Lipinski’s rule for compounds 4-17

| Comp. No. | TPSA<sup>a</sup> | Nrotb<sup>b</sup> | miLogP<sup>c</sup> | nOH-NH<sup>d</sup> | nO-N<sup>e</sup> | M. wt. | No. of violations |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|--------|------------------|
| 4         | 101.87          | 5               | 3.83            | 4               | 7               | 353.41 | 0                |
| 5         | 147.70          | 6               | 3.76            | 4               | 10              | 398.40 | 0                |
| 6         | 101.87          | 5               | 4.61            | 4               | 7               | 432.30 | 0                |
| 7         | 127.47          | 6               | 3.79            | 3               | 9               | 382.40 | 0                |
| 8         | 101.87          | 5               | 3.40            | 4               | 7               | 353.41 | 0                |
| 9         | 127.47          | 6               | 3.84            | 3               | 9               | 382.40 | 0                |
| 10        | 81.64           | 6               | 4.78            | 3               | 6               | 405.40 | 0                |
| 11        | 90.88           | 6               | 3.92            | 3               | 7               | 367.43 | 0                |
| 12        | 81.64           | 5               | 4.31            | 3               | 6               | 351.44 | 0                |
| 13        | 105.44          | 5               | 3.62            | 3               | 7               | 362.42 | 0                |
| 14        | 81.64           | 5               | 5.04            | 3               | 6               | 387.47 | 1                |
| 15        | 81.64           | 5               | 6.79            | 3               | 6               | 471.97 | 1                |
| 16        | 81.64           | 5               | 6.38            | 3               | 6               | 461.55 | 1                |
| 17        | 81.64           | 5               | 5.04            | 3               | 6               | 387.47 | 1                |
| Ampicillin| 112.73          | 4               | -0.87           | 4               | 7               | 349.41 | 0                |
| Fluconazole| 81.66          | 5               | -0.12           | 1               | 7               | 306.28 | 0                |

<sup>a</sup> TPSA: Topological polar surface area.
<sup>b</sup> Nrotb: Number of rotatable bonds.
<sup>c</sup> miLogP: The parameter of lipophilicity.
<sup>d</sup> nOH-NH: Number of hydrogen bond donor sites.
<sup>e</sup> nO-N: Number of hydrogen bond acceptor sites.
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HRMS of compound 4
HRMS of compound 5
HRMS of compound 6
HRMS of compound 7

MOUSTAFA_MG-II-49C_BWANG-ACCU_06142013_ESI-NEG01 108 (2.007) AM (Cen,2, 80.00, Ar,5000.0,554.26,1.0 9.87e3
$^1$H NMR spectrum of compound 7
HRMS of compound 8
$^1$H NMR spectrum of compound 8
$^{13}$C NMR spectrum of compound 8
HRMS of compound 9
HRMS of compound 10

MOUSTAVA_MG-II-55C_BWANG-ACCU_06112013_ESI-NEG01 23 (0.428) AM (Cen,2, 80.00, Ar,5000.0,554.26,1.00 3.88e4
$^1$HNMR spectrum of compound 10
HRMS of compound 11

MOUSTAFA_MG-II-52C_BWANG-ACCU_06112013_ESI-NEG 49 (0.912) AM (Cen,2, 80.00, Ar,5000.0, 554.26,1.00);
$^1$HNMR spectrum of compound 11
HRMS of compound 12
HRMS of compound 13
$^1\text{H}$ NMR spectrum of compound 13
HRMS of compound 14

MOUSTAFA_MG-II-66C_BWANG-ACCU_06112013_ESI-NEG 40 (0.742) AM (Cen,2, 80.00, Ar,5000.0,554.26,1.00); : 1.65e4
$^1$H NMR spectrum of compound 14
$^1$H NMR spectrum of compound 15
$^1$H NMR spectrum of compound 17