Design, synthesis, and antimicrobial evaluation of 1,4-dihydroindeno[1,2-c]pyrazole tethered carbohydrazide hybrids: Exploring their \textit{in silico} ADMET, ergosterol inhibition and ROS inducing potential

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CPF - Ciprofloxacin; MCZ - Miconazole

Supplementary Table S2: *In silico* ADME prediction of the most active hybrids (5d, 5g, 5j, 5k and 5p)
**Supplementary Table S3:** *In silico* Toxicity predictions of the most active hybrids (5d, 5g, 5j, 5k and 5p)

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CPF- Ciproflaxcin ; MCZ- Miconazole

**Experimental section**

**General materials and methods**

All the chemicals and reagents used in this study were obtained from Sigma-Aldrich, St. Louis, MO, USA, Lancaster and Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA), and were used without further purification. The reactions were monitored by TLC performed on silica gel glass plates containing 60 GF254, and visualized by using a UV light or iodine indicator. Column chromatography was executed using Merck 60-120 mesh silica gel. $^1$H NMR spectra were recorded on Bruker UXNMR/XWIN-NMR (300 MHz) or Innova Varian-VXRunity (400, 500 MHz) instruments. $^{13}$C NMR spectra were recorded on a Bruker UXNMR/XWIN-NMR (75, 100 and 125 MHz) instruments. Chemical shifts (δ) were reported in ppm downfield from an internal standard TMS and coupling constants are expressed in Hz. Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), m (multiplet) and bs (broad singlet). ESI spectra were recorded on Micro mass Quattro LC
using ESI+ software with a capillary voltage of 3.98 kV and an ESI mode positive ion trap detector. High-resolution mass spectra (HRMS) were recorded on a QSTAR XL hybrid MS-MS mass spectrometer. Melting points were determined with an electrothermal melting point apparatus and are uncorrected.

Scheme 1. Synthesis of new 1,4-Dihydroindeno[1,2-c]pyrazole- tethered carbohydrazide hybrids (5a-u)

**Reagents and conditions:** (i) NaOEt/EtOH, diethyl oxalate 5h, 0°C-rt, 70-81%; (ii) NH$_2$NH$_2$.2HCl ,EtOH, 3 h, reflux, 75-80%; (iii) Hydrazine hydrate, EtOH 3h, reflux, 85-90%; (iv) Substituted benzaldehydes, few drops of glacial acetic acid, EtOH 3-4h, reflux, 67-88%.
General procedure for the synthesis of substituted Diketo esters (2a-c) and substituted 1,4-dihydroindenopyrazole-3-carboxylates (3a-c)

These intermediates were prepared according to previously reported procedure. All the intermediates (2a-c and 3a-c) were characterized by suitable spectroscopic methods and were found to be identical to the previously reported ones.

General procedure for the synthesis of substituted 1,4-dihydroindenopyrazole-3-carbohydrazides (4a-c)

To the substituted indenopyrazole carboxylates (3a-c) (1.0 eq), was added hydrazine hydrate (99%) (4.0 eq) in ethanol at room temperature and refluxed for 3–4 h. The progress of the reaction was monitored by TLC using ethyl acetate and hexane (1: 1) as the solvent system. The solid precipitate obtained upon cooling was filtered off, washed with cold water and recrystallized from absolute ethanol to afford 1,4-dihydroindenopyrazole-3-carbohydrazides (4a-c) in excellent yields.

6-methoxy-1,4-dihydroindenopyrazole-3-carbohydrazide (4a)

White solid; 90.1% yield; mp: 155-156 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): δ 3.75 (s, 2H), 3.80 (s, 3H), 4.53 (bs, 2H), 6.85 (d, $J = 8.2$ Hz, 1H), 7.19 (s, 1H), 7.43 (d, $J = 8.2$ Hz, 1H), 9.33 (bs, 1H), 13.36 (bs, 1H); $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 28.0, 55.2, 104.4, 112.7, 123.5, 126.5, 130.2, 135.4, 139.6, 158.6, 159.3; MS (ESI): 245 [M+H]+.

1,4-dihydroindenopyrazole-3-carbohydrazide (4b)
Off white solid; 88.6% yield; mp: 161-162 °C; $^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$ 3.82 (s, 2H), 4.53 (bs, 2H), 7.27 (t, $J$ = 7.4, 8.6 Hz, 1H), 7.36 (t, $J$ = 7.3, 7.3 Hz, 1H), 7.56 (d, $J$ = 7.3 Hz, 1H), 7.65 (s, 1H), 9.36 (bs, 1H), 13.37 (bs, 1H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): $\delta$ 28.9, 118.7, 126.0, 126.4, 127.8, 129.9, 134.3, 147.8, 159.1; MS (ESI): 215 [M+H]+.

4.1.2.3 6-fluoro-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (4c)

Pale yellow solid; 85.7% yield; mp: 169-171 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 3.86 (s, 2H), 4.52 (bs, 2H), 7.17 (t, $J$ = 7.8, 8.1 Hz, 1H), 7.46 (d, $J$ = 8.0 Hz, 1H), 7.64 (s, 1H), 9.37 (bs, 1H), 13.36 (bs, 1H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): $\delta$ 28.3, 17.6, 117.6, 124.4, 127.3, 130.6, 134.6, 140.2, 159.2, 160.1; MS (ESI): 233 [M+H]+.

General procedure for the synthesis of (E)-N’-(substituted)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazides (5a-u)

Initially, equimolar quantities of appropriate hydrazide (4a-c) (1.0 eq), substituted benzaldehydes (1.0 eq) were dissolved in absolute ethanol (6 ml) followed by addition of catalytic amount of glacial acetic acid dropwise and the reaction was refluxed for 3-4 h. The progress of the reaction was monitored by TLC using ethyl acetate and hexane (1: 1) solvent system. Then the reaction was cooled to obtain the solid precipitate. The precipitate was filtered off, washed with ice cold water and recrystallized in small quantity of absolute ethanol to furnish 1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazides (5a-u) in good to excellent yields.

(E)-6-methoxy-N’-(3,4,5-trimethoxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5a)

Off White solid; 70.1% yield; mp: 175-176 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 3.66 (s, 2H), 3.71 (s, 3H), 3.83 (s, 6H), 3.84 (s, 3H), 6.88 (d, $J$ = 7.9 Hz, 1H), 6.99 (s, 1H), 7.05 (s, 1H), 7.20 (d, $J$ = 7.9 Hz, 1H), 7.49 (d, $J$ = 7.9 Hz, 1H), 8.45 (s, 1H), 11.76 (bs, 1H), 13.66 (bs, 1H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): $\delta$ 28.5, 55.9, 103.4, 106.3, 109.6, 110.8, 115.3, 121.2, 124.9, 126.5, 128.9, 132.7, 133.9, 140.4, 148.3, 152.4, 155.0; MS (ESI): 423 [M+H]+; HRMS (ESI) Calcd for C$_{22}$H$_{23}$N$_4$O$_5$ [M+H]+ 423.1663; found: 423.1670.
(E)-N’-(3,4-dimethoxybenzylidene)-6-methoxy-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5b)

Pale white solid; 71.8% yield; mp: 171-172 °C; 1H NMR (300 MHz, DMSO-d$_6$): δ 3.79 (s, 2H), 3.84 (s, 6H), 3.86 (s, 3H), 6.85 (d, J = 8.0 Hz, 1H), 6.99 (bs, 1H), 7.16 (s, 1H), 7.24 (s, 1H), 7.41 (t, J = 7.9, 8.1 Hz, 2H), 8.45 (s, 1H), 11.54 (bs, 1H), 13.54 (bs, 1H); 13C NMR (75 MHz, CDCl$_3$+DMSO-d$_6$): δ 26.8, 53.63, 53.98, 103.1, 106.8, 120.3, 126.1, 138.9, 147.4, 149.0, 158.3; MS (ESI): 393 [M+H]+; HRMS (ESI) Calcd for C$_{21}$H$_{21}$O$_4$N$_4$ [M+H]+ 393.1557; found: 393.1555.

(E)-N’-(4-hydroxybenzylidene)-6-methoxy-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5c)

Brown solid; 81.9 % yield; mp: 192-193 °C; 1H NMR (300 MHz, DMSO-d$_6$): δ 3.76 (s, 2H), 3.84 (s, 3H), 6.84 (d, J = 7.1 Hz, 3H), 7.21 (s, 1H), 7.41 (d, J = 8.1 Hz, 1H), 7.57 (d, J = 7.9 Hz, 2H), 8.40 (s, 1H), 7.97 (s, 1H), 11.34 (bs, 1H), 13.41 (bs, 1H); 13C NMR (75 MHz, DMSO-d$_6$): δ 27.0, 53.7, 103.4, 110.8, 114.1, 123.8, 124.9, 127.4, 130.4, 138.7, 146.9, 157.3, 158.0; MS (ESI): 449 [M+H]+; HRMS (ESI) Calcd for C$_{19}$H$_{17}$N$_3$O$_3$ [M+H]+ 349.1295; found: 349.1299.

(E)-N’-(4-hydroxy-3-methoxybenzylidene)-6-methoxy-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5d)

Light brownish solid; 79.3% yield; mp: 174-175 °C; 1H NMR (300 MHz, DMSO-d$_6$): δ 3.67 (s, 2H), 3.84 (s, 6H), 6.87 (t, J = 7.9 and 7.7 Hz, 2H), 7.05 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 8.5 Hz, 1H), 7.49 (s, 1H), 8.41 (s, 1H), 9.60 (s, 1H), 11.61 (bs, 1H), 13.41 (bs, 1H); 13C NMR (75 MHz, DMSO-d$_6$): δ 28.3, 55.2, 104.5, 108.9, 115.4, 122.1, 126.8, 147.9, 148.9, 158.6; MS (ESI): 379 [M+H]+; HRMS (ESI) Calcd for C$_{20}$H$_{19}$O$_4$N$_4$ [M+H]+ 379.1400; found: 379.1403.

(E)-N’-(4-bromobenzylidene)-6-methoxy-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5e)

White solid; 74.1% yield; mp: 190-192 °C; 1H NMR (300 MHz, DMSO-d$_6$): δ 3.66 (s, 2H), 3.82 (s, 3H), 6.88 (d, J = 6.8 Hz, 2H), 7.21 (d, J = 8.2 Hz, 1H), 7.47 (s, 1H), 7.67 (s, 3H), 8.50 (s, 1H), 11.83 (bs, 1H), 13.68 (bs, 1H); 13C NMR (75 MHz, DMSO-d$_6$): δ 28.2, 55.2, 104.5, 112.2,
(E)-N'-(4-chlorobenzylidene)-6-methoxy-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5f)

White solid; 81.5% yield; mp: 184-185 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 3.79 (bs, 5H), 6.99 (d, $J = 6.9$ Hz, 1H), 7.11 (d, $J = 6.9$ Hz, 1H), 7.38-7.42 (m, 3H), 7.68 (s, 1H), 7.98 (s, 1H), 8.44 (s, 1H), 11.60 (bs, 1H), 13.47 (bs, 1H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): $\delta$ 27.6, 55.5, 104.2, 110.7, 112.2, 113.3, 122.2, 123.3, 125.9, 127.9, 128.0, 130.7, 131.5, 132.2, 135.0, 139.1, 149.8, 158.8; MS (ESI): 367 [M+H]+; HRMS (ESI) Calcd for C$_{19}$H$_{16}$O$_2$N$_4$Cl [M+H]+ 367.0956; found: 367.0960.

(E)-6-methoxy-N'-(2,4,6-trifluorobenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5g)

Off White solid; 85.7% yield; mp: 202-204 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 3.67 (s, 2H), 3.83 (s, 3H), 6.89 (d, $J = 6.9$ Hz, 1H), 7.18 (d, $J = 8.1$ Hz, 1H), 7.47 (d, $J = 7.2$ Hz, 1H), 7.70 (s, 1H), 7.82 (s, 1H), 8.72 (s, 1H), 12.08 (bs, 1H), 13.71 (bs, 1H); $^{13}$C NMR (75 MHz, DMSO-d$_6$): $\delta$ 28.3, 55.2, 107.0, 104.5, 106.4, 112.1, 119.4, 126.4, 127.9, 132.0, 138.2, 139.3, 140.4, 145.0, 148.2, 150.1, 155.4, 158.3; MS (ESI): 387 [M+H]+; HRMS (ESI) Calcd for C$_{19}$H$_{14}$O$_2$N$_4$F$_3$ [M+H]+ 387.1063; found: 387.1066.

(E)-N'-(3,4,5-trimethoxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5h)

White solid; 68.7% yield; mp: 174-175 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 3.72 (s, 3H), 3.78 (s, 2H), 3.83 (s, 6H), 6.95 (d, $J = 7.9$ Hz, 1H), 7.10 (s, 1H), 7.18-7.33 (m, 2H), 7.47 (bs, 1H), 7.50 (d, $J = 6.6$ Hz, 1H), 8.34 (s, 1H), 11.47 (bs, 1H), 13.38 (bs, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$+DMSO-d$_6$): $\delta$ 28.3, 54.7, 58.9, 103.0, 105.1, 117.9, 124.5, 125.1, 125.6, 128.4, 130.5, 138.4, 146.2, 147.2, 152.11, 158.4; MS (ESI): 393 [M+H]+; HRMS (ESI) Calcd for C$_{21}$H$_{21}$O$_4$N$_4$ [M+H]+ 393.1557; found: 393.1561.

(E)-N'-(3,4-dimethoxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5i)
Off White solid; 67.6% yield; mp: 170-171 °C; $^1$H NMR (300 MHz, CDCl$_3$ + TFA-D): δ 3.90 (s, 2H), 3.99 (s, 6H), 7.00 (d, $J = 7.4$ Hz, 1H), 7.40 (d, $J = 7.9$ Hz, 1H), 7.51-7.73 (m, 4H), 7.99 (d, $J = 6.9$ Hz, 1H), 8.12 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$ + TFA-D): 30.3, 56.0, 104.7, 107.4, 109.2, 113.0, 120.0, 123.3, 127.0, 128.8, 132.0, 150.8, 152.6, 160.9; MS (ESI): 363 [M+H]$^+$; HRMS (ESI) Calcd for C$_{20}$H$_{19}$O$_3$N$_4$ [M+H]$^+$ 363.1451; found: 363.1454.

(E)-N'-(4-hydroxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5j)

Brown solid; 84.6% yield; mp: 201-203 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): δ 3.84 (s, 2H), 6.84 (d, $J = 7.9$ Hz, 2H), 7.24 (t, $J = 7.1$ and 7.4 Hz, 2H), 7.47 (d, $J = 7.9$ Hz, 1H), 7.52 (d, $J = 7.1$ Hz, 2H), 7.66 (d, $J = 6.7$ Hz, 1H), 8.37 (s, 1H), 9.64 (s, 1H), 11.22 (bs, 1H), 13.01 (bs, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$ + DMSO-d$_6$): δ 29.0, 115.3, 118.9, 125.1, 126.1, 126.5, 128.6, 148.1, 159.2; MS (ESI): 319 [M+H]$^+$; HRMS (ESI) Calcd for C$_{18}$H$_{15}$O$_2$N$_4$ [M+H]$^+$ 319.1189; found: 319.1188.

(E)-N'-(4-hydroxy-3-methoxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5k)

Pale brown solid; 81.1% yield; mp: 206-207 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): δ 3.77 (s, 2H), 3.84 (s, 3H), 6.84 (s, 1H), 7.01 (d, $J = 7.4$ Hz, 1H), 7.32-7.48 (m, 3H), 7.58-7.76 (m, 2H), 8.39 (s, 1H), 9.56 (s, 1H), 11.45 (bs, 1H), 13.56 (bs, 1H); $^{13}$C NMR (75 MHz, DMSO-d$_6$): 29.0, 55.5, 109.0, 115.4, 119.0, 122.0, 125.7, 126.2, 126.4, 147.9, 148.9; MS (ESI): 349 [M+H]$^+$; HRMS (ESI) Calcd for C$_{19}$H$_{17}$O$_3$N$_4$ [M+H]$^+$ 349.1295; found: 349.1294.

(E)-N'-(4-bromobenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5l)

White solid; 87.2% yield; mp: 199-201 °C; $^1$H NMR (300 MHz, DMSO-d$_6$): δ 3.81 (s, 2H), 7.29 (dd, $J = 7.4$ Hz, 3H), 7.49 (d, $J = 7.4$ Hz, 2H), 7.68-7.70 (m, 3H), 8.50 (s, 1H), 11.72 (bs, 1H), 13.43 (bs, 1H); $^{13}$C NMR (75 MHz, DMSO-d$_6$): δ 29.0, 104.8, 107.9, 119.0, 123.1, 126.2, 126.8, 128.8, 131.8, 148.3, 146.4; MS (ESI): 381 [M+H]$^+$; HRMS (ESI) Calcd for C$_{18}$H$_{14}$ON$_4$Br [M+H]$^+$ 381.0345; found: 381.0346.
(E)-N’-(4-chlorobenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5m)
White solid; 77.7% yield; mp: 194-196 °C; ¹H NMR (300 MHz, DMSO-d₆): δ 3.83 (s, 2H), 7.24 (dd, J = 7.4 Hz, 2H), 7.28-7.36 (m, 2H), 7.48 (d, J = 7.4 Hz, 2H), 7.61 (d, J = 6.9 Hz, 2H), 8.48 (s, 1H), 11.60 (bs, 1H), 13.47 (bs, 1H); ¹³C NMR (75 MHz, DMSO-d₆): δ 28.1, 111.2, 119.2, 120.0, 122.3, 123.9, 125.8, 126.6, 128.4, 129.4, 132.0, 133.9, 138.3, 147.8, 159.8; MS (ESI): 337 [M+H]+; HRMS (ESI) Calcd for C₁₈H₁₄ON₄Cl [M+H]+ 337.0850; found: 337.0852.

(E)-N’-(2,4,6-trifluorobenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5n)
Off white solid; 85.4% yield; mp: 211-212 °C; ¹H NMR (300 MHz, DMSO-d₆): δ 3.76 (s, 2H), 7.35 (dd, J = 7.4 Hz, 2H), 7.58-7.75 (m, 3H), 7.82-7.85 (m, 1H), 8.73 (s, 1H), 12.11 (bs, 1H), 13.80 (bs, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ 29.2, 109.8, 114.0, 125.5, 127.8, 127.8, 129.9, 134.5, 138.9, 148.9, 150.9, 160.1, 162.5; MS (ESI): 357 [M+H]+; HRMS (ESI) Calcd for C₁₈H₁₂F₃N₄O₄ [M+H]+ 357.0958; found: 357.0957.

(E)-6-fluoro-N’-(3,4,5-trimethoxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5o)
Off white solid; 68.6% yield; mp: 198-199 °C; ¹H NMR (300 MHz, DMSO-d₆): δ 3.77 (s, 3H), 3.78 (s, 6H), 3.92 (s, 2H), 6.98 (s, 1H), 7.08 (s, 1H), 7.29 (t, J = 6.0 and 9.0 Hz, 1H), 7.48 (d, J = 9.0 Hz, 2H), 8.44 (s, 1H), 11.76 (bs, 1H), 13.59 (bs, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ 29.4, 55.8, 60.04,104.0,105.5,113.9, 120.3, 126.8, 127.6, 130.0, 140.1, 147.2, 153.1, 158.1, 161.4; MS (ESI): 411 [M+H]+; HRMS (ESI) Calcd for C₂₁H₂₀F₃N₄O₄ [M+H]+ 411.1463; found: 411.1474.

(E)-N’-(3,4-dimethoxybenzylidene)-6-fluoro-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5p)
Off white solid; 70.8% yield; mp: 206-207 °C; ¹H NMR (300 MHz, DMSO-d₆): δ 3.82 (s, 6H), 3.95 (s, 2H), 7.00 (t, J = 6.6 and 7.1 Hz, 1H), 7.14-7.29 (m, 2H), 7.33 (d, J = 8.9 Hz, 1H), 7.48 (d, J = 9.9 Hz, 1H), 7.59 (t, J = 5.5 and 6.3 Hz, 1H), 8.44 (s, 1H), 11.64 (bs, 1H), 13.67 (bs,
(E)-6-fluoro-N’-(4-hydroxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5q)
Brown solid; 87.1% yield; mp: 215-217 °C; ¹H NMR (400 MHz, DMSO-d₆): δ 3.81 (s, 2H), 6.84 (d, J = 7.7 Hz, 2H), 7.21 (s, 2H), 7.45 (d, J = 8.6 Hz, 1H), 7.50-7.72 (m, 3H), 8.41 (s, 1H), 9.96 (s, 1H), 11.56 (bs, 1H), 13.61 (bs, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ 29.4, 113.6, 115.6, 119.9, 124.7, 125.4, 126.7, 127.7, 128.7, 138.8, 147.7, 157.9, 159.2, 162.5; MS (ESI): 337 [M+H]+; HRMS (ESI) Calcd for C₁₈H₁₄O₂N₄F [M+H]+ 337.1095; found: 337.1093.

(E)-6-fluoro-N’-(4-hydroxy-3-methoxybenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5r)
Pale brown solid; 83.2% yield; mp: 208-210°C; ¹H NMR (300 MHz, DMSO-d₆): δ 3.98 (s, 5H), 6.85 (s, 1H), 7.06 (dd, J = 8.5 Hz, 2H), 7.28 (d, J = 7.4 Hz, 2H), 7.56-7.74 (m, 1H), 8.40 (s, 1H), 9.46 (s, 1H), 11.46 (bs, 1H), 13.53 (bs, 1H); ¹³C NMR (75 MHz, DMSO-d₆): δ 27.0, 54.0, 101.6, 108.4, 119.6, 121.2, 122.1, 125.4, 126.3, 127.5, 130.1, 132.8, 138.2, 139.8, 147.0, 149.8, 150.1, 155.3; MS (ESI): 367 [M+H]+; HRMS (ESI) Calcd for C₁₉H₁₆O₃N₄F [M+H]+ 367.1201; found: 367.1204.

(E)-N’-(4-bromobenzylidene)-6-fluoro-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5s)
Off White solid; 82.9% yield; mp: 212-213°C; ¹H NMR (300 MHz, DMSO-d₆): δ 3.78 (s, 2H), 7.22 (s, 1H), 7.47 (d, J = 7.4 Hz, 1H), 7.66 (s, 2H), 7.72 (d, J = 8.5 Hz, 1H), 7.83 (d, J = 8.5 Hz, 1H), 8.70 (s, 1H), 11.85 (bs, 1H), 13.71 (bs, 1H); ¹³C NMR (100 MHz, CDCl₃, DMSO-d₆): δ 26.9, 109.8, 112.3, 117.5, 120.6, 121.2, 121.8, 124.4, 125.3, 128.1, 129.1, 130.9, 132.7, 137.2, 146.3, 161.0; MS (ESI): 399 [M+H]+; HRMS (ESI) Calcd for C₁₉H₁₆ON₄BrF [M+H]+ 399.0251; found: 399.0250.

(E)-N’-(4-chlorobenzylidene)-6-fluoro-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5t)
Off White solid; 80.8% yield; mp: 210-212°C; $^1$H NMR (400 MHz, DMSO-d$_6$): δ 3.76 (s, 2H), 7.19 (dd, $J = 8.5$ Hz, 1H), 7.47 (d, $J = 9.0$ Hz, 1H), 7.52 (t, $J = 8.1$ and 8.1 Hz, 2H), 7.59 (d, $J = 8.5$ Hz, 1H), 7.69 (d, $J = 8.9$ Hz, 2H), 8.52 (s, 1H), 11.82 (bs, 1H), 13.70 (bs, 1H); $^{13}$C NMR (75 MHz, DMSO-d$_6$): δ 29.2, 114.0, 120.4, 123.9, 125.6, 128.8, 136.2, 141.0, 147.5, 151.0, 159.7; MS (ESI): 355 [M+H]$^+$; HRMS (ESI) Calcd for C$_{18}$H$_{13}$ON$_4$ClF [M+H]$^+$ 355.0756; found: 355.0758.

(E)-6-fluoro-N’-(2,4,6-trifluorobenzylidene)-1,4-dihydroindeno[1,2-c]pyrazole-3-carbohydrazide (5u)

Off White solid; 86.7% yield; mp: 209-212 °C; $^1$H NMR (400 MHz, DMSO-d$_6$): δ 3.80 (s, 2H), 6.85- 6.87 (m, 1H), 7.29 (s, 1H), 7.61 (t, $J = 5.0$ and 5.0 Hz, 2H), 7.69 (m, 1H), 8.72 (s, 1H), 11.89 (bs, 1H), 13.82 (bs, 1H); $^{13}$C NMR (75 MHz, DMSO-d$_6$): δ 29.0, 55.5, 109.0, 115.4, 119.0, 122.0, 125.7, 126.2, 126.4, 147.9, 148.9; MS (ESI): 375 [M+H]$^+$; HRMS (ESI) Calcd for C$_{18}$H$_{13}$ON$_4$F$_4$ [M+H]$^+$ 375.0863; found: 375.0870.

Biology

Antimicrobial activity

All the test pathogenic strains were procured from MTCC, CSIR-IMTECH, Chandigarh, India. These strains were used to evaluate the antibacterial activity of the synthesized AD derivatives by employing agar well diffusion method. These pathogens equivalent to 0.5 Mc Farland were spread on Mueller-Hinton agar plates. Later, 6mm diameter wells were made on these pathogen spread using a sterile borer. Later, the synthesized derivatives (5a-5u) at a 125 - 0.97 μg/mL dose range were loaded into the wells. Ciprofloxacin at a dose 125 - 0.97 μg/mL was positive control and DMSO was negative control. After incubation for 24h at 37 °C, Minimum inhibitory concentration is considered as the well with least concentration of synthesized derivatives exhibiting a clear zone of inhibition. Experiments were performed in triplicates and mean values of MICs are presented.
Antifungal activity

The antifungal activity in terms of MIC was performed by using the *Candida* strains procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. This assay was performed by agar well diffusion method. All the pre-cultured *Candida* strains were spread on Muller Hinton Agar plates. Wells were made in Muller Hinton Agar plates loaded with AD-series compounds dissolved in 10% DMSO at a 125 - 0.97μg/mL and Miconazole standard solution at a 125 - 0.97 μg/mL dose range. DMSO was employed as a negative control. After 24h incubation at 37°C for 24hr, wells showing inhibition zone at least concentration of test compound was considered as MIC. Entire experiments were carried out in duplicates to represent the mean values.

Minimum bactericidal concentration (MBC)/ assay

Mueller Hinton broth was used to culture the test pathogenic strains Pathogenic bacterial strains *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453 and *Klebsiella planticola* MTCC 530 at 37 °C. Micro-centrifuge tubes were used for performing the minimum bactericidal concentration assay. A concentration range of test compounds from 0 to 125 μg/mL was prepared in Mueller Hinton broth. 100 μL of overnight culture of each pathogen was added to each concentration of test compound to get 0.5 McFarland standard or 1.5 × 10^8 cfu mL^-1 and incubated for 24hr at 37 °C. Then 10μL sample from each tube was seeded onto the Mueller Hinton agar plates to examine the growth at each concentration. The minimum concentration of a test compound required to kill the corresponding pathogenic bacterial strain was considered as MBC. Mean values are presented from all the experiments performed in duplicates.

Minimum fungicidal concentration (MFC)

Fungicidal activity of the most active derivatives with promising anticandida activity was evaluated in terms of MIC values. For this microcentrifuge tubes of 2mL were used. Test compounds were dissolved in Muller Hinton Broth to achieve a concentration range of 125 - 0.97
μg/mL. 100 μL of each *Candida* strain was added and allowed for incubation at 37 °C for 24 hr. Later Muller Hinton agar plates were seeded with 10μL of each test compound treated suspension to observe the effect of synthesized derivatives on the growth of *Candida* strains. MFC is derived from the well showing zone of the least concentration of test compound that can kill the *Candida* strain, after 24 hr incubation at 37 °C.

Mean values are represented from all the above experiments carried out in duplicates. All the experiments were performed in triplicates and mean values are considered for calculating the standard deviations.

**Ergosterol quantification assay**

The total intracellular sterol content from *Candida albicans* were measured using Brevik and Owades protocol with some modifications incorporated. Muller Hinton Broth was treated with various concentrations of 5d and 5g (0, 2, 4, 8, 16 µg mL⁻¹). Later, this broth was inoculated with single colonies of test *Candida* strains and incubated at 30 °C for 20 h at 200 rpm. This was followed by centrifugation of cells at 8200 ×g for 15 min and by washing the pellet twice with sterile distilled water. The total wet weight of pellets of individual *Candida* member was measured. To each *Candida* pellet, 5 mL of 25% alcoholic potassium hydroxide solution was mixed by vigorous shaking. These suspensions were collected in sterile tubes and incubated at 90 °C for 70 min. After allowing these suspensions to stay at room temperature, sterile distilled water and n-heptane (1:3) was added and mixed for 5 min by vortexing and mixing for the extraction of intracellular sterols into the heptane layer. The sterols from the heptane layer were collected and stored at -20 °C for 30 h, after which the stored suspension was diluted with absolute ethanol in 1:5 ratio. This ethanolic suspension was scanned at a range of 230-310 nm employing UV-visible spectrophotometer for the four characteristic peaks representing the ergosterol and dehydroergosterol.

The following formula was employed for the calculation of ergosterol percentage of each test *Candida* strain as percentage of wet weight of the cell pellet.

\[
\% \text{ergosterol} + \% \text{24(28)DHE} = \frac{[(A_{281.5}/290) \times F]}{\text{pellet weight}}
\]
\% 24(28) DHE = \[(A_{230}/518) \times F\] / pellet weight

F = Dilution factor for dissolving in ethanol

290 = E value (percent per centimeter) for crystalline ergosterol

518 = E value (percent per centimeter) for crystalline dehydroergosterol

All the experiments were performed in triplicates and mean values were considered for calculating the standard deviations.

**ROS quantification in *Candida albicans***

Since the synthesized derivatives showed promising and broad spectrum *Candida* biofilm inhibition, the mechanistic role of the derivatives was explored. Most of the antifungals generate intracellular ROS in *Candida* species that kills the yeast cells. Considering this fact, the intracellular ROS accumulated by 5d and 5g was measured in *C. albicans* MTCC 3017 by 2', 7'-dichlorofluorescein diacetate (DCFH-DA)-dependant fluorometric assay. The *C. albicans* MTCC 3017 biofilms developed in 96-well microtiter plate at 37 °C for 24 h. These biofilms were treated with various concentrations of the most active derivatives 5d and 5g at a concentration range of 0, 5, 10, 15, 20 and 25 µg µg/mL for 24 hr to observe any effect on biofilm formation or disruption. Control was established from untreated *Candida* cells. Followed by the incubation with the synthesized derivatives, all the experimental Candida groups were treated with 10 µM of DCFH-DA with as incubation in dark for 24 h. The fluorescent product that would be proportional to the amount of ROS generated was measured at excitation and emission wavelengths of 485 nm and 535 nm on Infinite M200Pro microtiter plate reader (Tecan). All the measurements were performed in triplicates and the mean values were considered for the calculation of standard deviation. The resulting fluorescence intensities resulted from the treatment with various concentrations of 5d and 5g were plotted.

**In vitro Cytotoxicity assay**
The cell viability or non-toxic nature of the active compounds was examined against normal human lung cell line MRC5 (ATCC-CCL 171) using MTT assay. The MRC5 cells were grown for a period of 24 h in 96-well microplates. After incubation, the cells were treated with different concentrations of test compounds for 48 h. Further, the cells were incubated with MTT reagent (250 μg/mL) for 2 h. After incubation, the medium was substituted with 100 μL of DMSO and the plates were measured at 570 nm on a microplate reader. Miconazole and DMSO were used as positive and negative controls, respectively. All the experiments were performed in triplicates and the results are represented in mean ± S.D.

**Molecular docking Studies**

The heme proteins (CYP51), sterol 14-α-demethylases (PDB: 3GW9 and 1EA1) of parasite (*Trypanosoma brucei*), bacteria (*Mycobacterium tuberculosis*) and fungi (*Candida Albicans*) (PDB: 4UYM) along with their co-crystal ligands were retrieved from protein data bank. All the demethylases were prepared and their energy was minimized by protein preparation wizard and azole binding site exists in their respective cavities were employed for current docking investigation. The ligands were sketched using Maestro 11.6 panel and prepared by Ligprep module. The extra precision docking protocol implemented in Glide module of Schrodinger LLC were employed for receptor-ligand binding studies.
Representative Spectra:

'H NMR of 5d
$^{13}$C NMR OF 5d
HRMS of 5d
$^{1}$H NMR of 5g
$^{13}$C NMR of 5g
HRMS of 5g

$C_{19}H_{14}O_{2}N_{4}F_{3} = 387.10634$

13.5 RDBE
0.82768 ppm
$^{1}$H NMR of 5k
$^{13}$C NMR of 5k
HRMS of 5k
$^1$H NMR of 5n
$^{13}$C NMR of 5n
HRMS of 5n
HPLC spectrum of compound 5d
HPLC spectrum of compound 5g

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