Novel phthalimide based analogues: design, synthesis, biological evaluation, and molecular docking studies

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ABSTRACT
Pyrazolylphthalimide derivative 4 was synthesized and reacted with different reagents to afford the target compounds imidazopyrazoles 5-7, pyrazolopyrimidines 9, 12, 14 and pyrazolotriazines 16, 17 containing phthalimide moiety. The prepared compounds were established by different spectral data and elemental analyses. Additionally, all synthesized derivatives were screened for their antibacterial activity against four types of Gram +ve and Gram-ve strains, and for antifungal activity against two fungi micro-organisms by well diffusion method. Moreover, the antiproliferative activity was tested for all compounds against human liver (HepG-2) cell line in comparison with the reference vinblastine. Moreover, drug-likeness and toxicity risk parameters of the newly synthesized compounds were calculated using in silico studies. The data from structure-activity relationship (SAR) analysis suggested that phthalimide derivative bearing 3-amino-pyrazolone moiety, 4 illustrated the best antimicrobial and antitumor activities and might be considered as a lead for further optimization. To investigate the mechanism of the antimicrobial and anticancer activities, enzymatic assay and molecular docking studies were carried out on E. coli topoisomerase II DNA gyrase B and VEGFR-2 enzymes.

Introduction
Nowadays, the most serious public health problems in the world are cancer and infectious diseases. The evidence of multi-drug resistant microbial pathogens due to extensive use of antibiotics has been appeared and stimulated the search for discovery of new safer, potent, and resistance-free antimicrobial agents. Moreover, the research for novel, selective and more potent antitumor agents is still a vital challenge for biologists and medicinal chemists.

Thalidomide is known as a multi-target drug that affects several cellular processes, including peptidase inhibition, cyclooxynase COX inhibition, glucosidase inhibition and androgen receptor antagonism. Research studies on the structure-activity relationship (SAR) of the metabolites and analogues of thalidomide have revealed that the phthalimide ring system is an essential pharmacophoric fragment. Phthalimide (isoindoline-1,3-dione) has usually been employed in the design of potential antitumour, immunomodulatory, antiangiogenic, anti-microbial and anti-inflammatory drug candidates. Further, heterocyclic hits are of considerable utility in synthetic medicines or pesticides and biochemical effects. Heterocycles containing pyrazole, imidazo[1,2-b]-pyrazole, pyrazolopyrimidines, pyrazolo-triazine scaffolds exhibit versatile biological properties such as anti-inflammatory, antifungal, antioxidant, antitumor and immunosuppressive agents. Hence, molecular hybridization strategy via introduction of different pharmacophoric fragments might improve the biological activity of phthalimide derivatives.

Bearing in mind our program in the synthesis of biologically active heterocyclic compounds and the molecular pharmacophores outlined in Figure 1 and their structural requirements, some phthalimide derivatives were designed after exploring molecular hybridization approaches with pyrazole, imidazo[1,2-b]-pyrazole, pyrazolopyrimidine, pyrazolo-triazine moieties (Figure 1). All the newly prepared phthalimide derivatives were subjected for evaluation of both antimicrobial and anticancer activities with the study of their Drug-Likeness and Toxicity parameters. Furthermore, in-vitro enzyme assay of the most potent derivative was performed against E. coli topoisomerase II DNA gyrase B and VEGFR-2 enzymes, followed by molecular docking studies to get a distinct insight about the interactions and binding mode in the active sites of these enzymes.

Experimental
Chemistry
All melting points were determined on a Gallenkamp apparatus and were uncorrected. The IR spectra were measured on a Pye-Unicam SP300 instrument in potassium bromide discs. The 1H-NMR
and 13C-NMR spectra were recorded in DMSO-d$_6$ at 400, 500 MHz on JEOL and Broker NMR spectrometer (δ, ppm) using TMS as an internal standard. Mass spectra were obtained on JEOL JMS600 H Root mass spectrometer at 70 ev. Elemental analyses were carried out by the Micro analytical Center of Cairo University, Giza, Egypt.

The antimicrobial and anticancer activities were carried out in the Medical Mycology Laboratory of the Regional Center for Mycology and Biotechnology of Al-Azhar University, Cairo, Egypt.

Ethyl 2-cyano-2-(2-(1,3-dioxoisoindolin-2-yl)hydrazono)acetate (3)

A solution of 2-aminoisoindoline-1,3-dione (1) (10 mmol) in HCl (3 ml) and water (2 ml) was stirred in ice bath and diazotized with NaNO$_2$ (0.3 g, in 5 ml H$_2$O). The cold diazonium solution was added to ethyl cyanoacetate (10 mmol) in EtOH (20 ml) containing CH$_3$COONa (2 g), was stirred for 2 h. The formed solid was collected by filtration, dried and crystallized from toluene to obtain 3, (87%) as pale red crystals, m.p. 128–129°C; IR (KBr) ν cm$^{-1}$ 3355 (NH), 3060 (CH-arom.), 2936 (CH-aliph.), 2225 (CN), 1735, 1713, 1660 (3C=O); $^1$H NMR (DMSO-d$_6$) δ = 1.22–1.29 (t, J = 7.2 Hz, 3H, OCH$_2$CH$_3$), 4.17–4.27 (q, J = 7.2 Hz, 2H, CH$_2$, OCH$_2$CH$_3$), 7.73–7.80 (m, 4H, Ar-H), 11.12 (s, 1H, NH); 13C NMR (DMSO-d$_6$): 13.5, 60.6, 110.2, 115.3, 124.8, 131.4, 133.3, 162.1, 165.8. Analysis for C$_{13}$H$_{10}$N$_4$O$_4$ (286.24): Calculated: C, 54.55; H, 3.52; N, 19.57%. Found: C, 54.77; H, 3.76; N, 19.79%.

2-(2-(3-Amino-5-oxo-1H-pyrazol-4(5H)-ylidene)hydrazinyl)isoindoline-1,3-dione (4)

A mixture of compound 3 (2.9 mg, 10 mmol) and NH$_2$NH$_2$.H$_2$O (2 ml) in EtOH (30 ml) was refluxed for 4–6 h, concentrated then cooled. The obtained precipitate was filtered off, dried and crystallized from ethanol to give 4 as brown crystals, yield 83%; m.p. 289–290°C. IR (KBr): ν cm$^{-1}$ 3422, 3375 and 3268, 3140 (NH$_2$ and 2NH), 3080 (CH-arom.), 2945 (CH-aliph.), 1739, 1681, 1661 (3 C=O), 1610 (C=N); $^1$H NMR (DMSO-d$_6$) δ = 7.75–7.93 (m, 5H, Ar-H + NH), 8.98 (s, 2H, NH$_2$), 11.52 (s, 1H, NH) ; 13C NMR (DMSO-d$_6$): 122.4, 132.6, 135.5, 136.2, 142.8, 163.7, 167.1; MS: m/z = 272 [M$^+$]. Analysis for C$_{11}$H$_8$N$_6$O$_3$ (272.22): Calculated: C, 48.53; H, 2.96; N, 30.87%. Found: C, 48.74; H, 2.75; N, 30.66%.

Figure 1. Representative examples of antimicrobial and anticancer agents and structural rationalization of the newly designed compounds 4–17.
General procedure for the synthesis of imidazo[1,2-b]pyrazole derivatives (5–7)

A mixture of compound 4 (2.7 mg, 10 mmol) and each of ethyl chloroacetate, chloroacetonitrile and phenacyl bromide (10 mmol) was refluxed in DMF (30 ml) containing (0.015 mmol) of NaOH for 5 h. The mixture was poured onto ice and acidified with dilute HCl. The solid obtained were composed by filtration and crystallized from the appropriate solvent to give 5, 6, and 7, respectively.

2-[[6-Hydroxy-2-oxo-2,3-dihydro-1H-imidazo[1,2-b]pyrazol-7-yl]diazenyl]-isoindoline-1,3-dione (5)

It was obtained as pale yellow crystals from benzene; yield 73%; m.p. 157–158 °C; IR (KBr); ν cm⁻¹ 3500 (OH), 3261 (NH), 3080 (CH- arom.), 2984 (CH-aliph.), 1735, 1685, 1667 (3C=O), 1612 (C=N), 1515 (N=N); ¹H NMR (DMSO-d₆) δ = 4.62 (s, 2H, CH₂), 7.70-7.84 (m, 4H, Ar-H), 9.07 (s, 1H, NH), 11.52 (hump, 1H, OH); ¹³C NMR (DMSO-d₆): 65.1, 90.5, 123.2, 133.5, 148.0, 160.3, 169.4, 181.6. Analysis for C₁₆H₁₀N₈O₃: Calculated: C, 50.01; H, 2.58; N, 26.92%. Found: C, 50.22; H, 2.80; N, 26.71%.

2-[[2-Amino-6-hydroxy-3H-imidazo[1,2-b]pyrazol-7-yl]diazenyl]-isoindoline-1,3-dione (6)

It was obtained as pale yellow crystals from toluene; yield 69%; m.p. 180–182 °C; IR (KBr): ν cm⁻¹ 3500 (OH), 3328, 3144 (NH₂), 3086 (CH-arom.), 2954 (CH-aliph.), 1740, 1709 (2C=O), 1495 (N=N); ¹H NMR (DMSO-d₆) δ = 4.02 (s, 2H, CH₂), 7.72–7.88 (m, 4H, Ar-H), 8.52 (s, 2H, NH₂), 11.40 (hump, 1H, OH); ¹³C NMR (DMSO-d₆): 61.3, 104.1, 123.4, 132.0, 133.7, 145.2, 162.1, 167.5, 169.9. Analysis for C₁₆H₁₀N₈O₃: Calculated: C, 50.16; H, 2.91; N, 31.50%. Found: C, 50.37; H, 2.69; N, 31.72%.

2-[[6-Hydroxy-2-phenyl-1H-imidazo[1,2-b]pyrazol-7-yl]diazenyl]-isoindoline-1,3-dione (7)

It was obtained as pale yellow crystals from toluene; yield 76%; m.p. 196–198 °C; IR (KBr): ν cm⁻¹ 3504 (OH), 3237 (NH), 3078 (CH-arom.), 2942 (CH-aliph.), 1745, 1710 (2C=O), 1488 (N=N); ¹H NMR (DMSO-d₆) δ = 7.35–8.49 (m, 10H, Ar-H + CH-imidazolone), 12.02 (s, 1H, NH), 12.75 (s, 1H, OH); ¹³C NMR (DMSO-d₆): 101.2, 120.0, 123.4, 127.5, 129.7, 130.3, 131.1, 132.4, 133.8, 140.2, 143.0, 160.6, 166.7, 169.5; MS: m/z = 372 [M⁺]. Analysis for C₁₅H₁₀N₈O₃: Calculated: C, 61.29; H, 3.25; N, 22.57%. Found: C, 61.50; H, 3.47; N, 22.78%.

5-Amino-3-[[1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl]diazenyl]-2-hydroxy-7-(methylthio)pyrazolo[1,5-a]pyrimidine-6-carbonitrile (9)

A mixture of compound 4 (2.7 mg, 10 mmol) and 2-(bis(methylthio)methylene)malononitrile (1.7 mg, 10 mmol) in DMF (30 ml), in the presence of anhydrous K₂CO₃ (1.0 g) refluxed for 7 h. After cooling, the solid produced was left at room temperature for 2 h with stirring. The solid result, produced in each case, was filtered off and crystallized from the ethyl acetate to yield 16 and 17, respectively.

7-Amino-3-[[1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl]diazenyl]-2-hydroxy-7-[1,5-alpyrimidine-6-carbonitrile (12)

A mixture of 10 (3.3 mg, 10 mmol) and cyanoacetic acid or malononitrile (10 mmol) in EtOH (30 ml) containing TEA (0.5 ml) was refluxed for 4 h. After cooling, the resulted solid was composed by filtration and crystallized from EtOH to yield compound 12 as brown crystals (66%), m.p. 302–303 °C; IR (KBr): ν cm⁻¹ 3500 (OH), 3386, 3248 (NH₂), 3078 (CH-arom.), 2955 (CH-aliph.), 2220 (CN), 1757, 1712 (2C=O), 1496 (N=N); ¹H NMR (DMSO-d₆) δ = 7.60–7.86 (m, 4H, Ar-H), 8.11 (s, 1H, CH=N), 9.27 (s, 2H, NH₂), 12.63 (s, 1H, OH); ¹³C NMR (DMSO-d₆): 93.6, 101.2, 115.1, 123.6, 132.4, 133.0, 147.7, 160.4, 162.7, 168.1, 170.0. Analysis for C₁₉H₁₂N₆O₃: Calculated: C, 51.73; H, 2.32; N, 32.17%. Found: C, 51.94; H, 2.55; N, 32.38%.

2-(7-Amino-6-benzoyl-2-hydroxypyrazolo[1,5-a]pyrimidin-3-yl]-1H-isooindole-1,3(2H)-dione (14)

A mixture of compound 10 (3.3 mg, 10 mmol) and 3-oxo-3-phenylpropanenitrile (1.5 mg, 10 mmol) in glacial acetic acid (30 ml) was heated under reflux for 4 h. The solid production acquire after cooling was filtered off and crystallized from benzene to give compound 14 as brown crystals (57%), m.p 295–297 °C; IR (KBr): ν cm⁻¹ 3502 (OH), 3382, 3241 (NH₂), 3076 (CH-arom.), 2950 (CH-aliph.), 1746, 1708, 1664 (3C=O), 1500 (N=N); ¹H NMR (DMSO-d₆) δ = 7.48–7.89 (m, 9H, Ar-H), 8.05 (s, 1H, CH₂), 9.14 (s, 2H, NH₂), 12.71 (s, 1H, OH); ¹³C NMR (DMSO-d₆): 100.6, 119.1, 123.8, 128.3, 129.5, 132.4, 133.1, 134.2, 147.8, 160.7, 162.0, 164.1, 170.7, 191.3; MS: m/z = 472 (75%) [M⁺], 105 (100%) B.P. Analysis for C₁₉H₁₄N₄O₄: Calculated: C, 59.02; H, 3.07; N, 22.94%. Found: C, 59.23; H, 3.28; N, 22.72%.

General procedure for the synthesis of pyrazolo[5,1-c][1,2,4]triazine derivatives 16 and 17

To a cold solution (0–5 °C) of malononitrile, ethyl acetoacetate, and 3-iminobutanenitrile (10 mmol) in EtOH (30 ml) containing CH₂COONA (2g), a solution of diazonium chloride 15 (prepared from 10mmol of 4 and the appropriate quantities of conc. HCl and sodium nitrate) was added. The reaction combination, in each case, was left at room temperature for 2h with stirring. The solid result, produced in each case, was filtered off and crystallized from the ethyl acetate to yield 16 and 17, respectively.
4-Amino-8-[(1,3-dioxo-1,3-dihydro-2H-isindol-2-yl)diazenyl]-7-hydroxy-pyrazolo[5,1-c][1,2,4]triazine-3-carbonitrile (16)

It was obtained as buff crystals from dioxane; yield 60%; m.p. >300 °C; IR (KBr): ν cm⁻¹ 3500 (OH), 3070 (CH-arom.), 2952 (CH-ariph.), 1748, 1718, 1707 (C=O), 1492 (N=N); ¹H NMR (DMSO-d₆) δ = 1.21–1.28 (t, J = 7.2 Hz, 3H, OCH₂CH₃), 2.25 (s, 3H, CH₃), 4.16–4.26 (q, J = 7.2 Hz, 2H, CH₂, OCH₂CH₃), 7.62–7.83 (m, 3H, Ar-H), 13.15 (s, 1H, OH); ¹³C NMR (DMSO-d₆) δ 12.7, 14.1, 60.9, 100.2, 123.4, 132.3, 133.1, 148.3, 151.8, 155.2, 161.0, 169.6, 170.8. Analysis for C₁₇H₁₃N₇O₅ (395.33): Calculated: C, 51.65; H, 3.31; N, 24.80%.

Results and discussion

Chemistry

The target derivatives which obtained are showed in Schemes 1–3 based on the synthetic strategies. Synthesis of the precursor hydrazone 3 was achieved by diazotization of N-aminophthalimide (1)⁴⁶, followed by coupling with ethyl cyanoacetate at room temperature in sodium acetate (Scheme 1). The spectral data confirmed that this compound exists in the hydrazone⁴⁹ form 3b, as the ¹H-NMR and ¹³C NMR spectra (Scheme 1).

2-(2-(3-Amino-5-oxo-1H-pyrazol-4-(5H)-ylidene)hydrazinyl)-isindoline-1,3-dione (4) was synthesized via cyclization of 3 with NH₂NH₂·H₂O under reflux in ethanol. The reaction of 4 with ethyl chloroacetate or chloroacetonitrile in NaOH/DMF solution under reflux yielded the corresponding compounds 5 and 6, respectively. Treatment of 4 with phenacylbromide afforded imidazopyrazole derivative 7. 2-(Bis(methylthio)ethyl)methylene)malononitrile was reacted with compound 4 in the presence of K₂CO₃ as a catalyst in DMF under reflux to afford compound 9. Condensation of 4 with DMF-DMA in dry dioxane under reflux afforded N,N-dimethyl-mido-formamide derivative 10, which was treated with cyanothioacetamide in ethanol/piperidine to afford compound 12. Moreover, the reaction of 10 with benzoylacetoneitrile in refluxing glacial acetic acid yielded product 14 (Scheme 2).

Moreover, aminopyrazole is used for formation of pyrazolotriazine derivatives through diazotization and coupling with active methylene compounds⁴⁰. Aminopyrazole 4 can be diazotized with NaNO₂ and HCl to give the diazonium salt (intermediate) 15, which coupled with malononitrile and ethyl acetoacetate in ethanol to yield the pyrazolo[5,1-c][1,2,4]triazine derivatives 16 and 17, respectively (Scheme 3).
Scheme 1. Synthesis of amino pyrazole derivative.

Scheme 2. Synthesis of imidazopyrazole and pyrazolopyrimidine derivatives.
Table 1. Diameter of inhibition zone (Mean ± SEM) (mm)

| Compd. | Gram-ve Bacteria | Gram +ve Bacteria | Fungi |
|--------|------------------|------------------|-------|
|        | S. pneumonia RCMB 010010 | B. subtilis RCMB 010067 | P. aeruginosa RCMB 010043 | E. coli RCMB 010052 | A. fumigatus RCMB 002568 | C. albicans RCMB 005036 |
| 3      | 18.3 ± 0.7       | 17.3 ± 0.6       | 15.3 ± 0.7 | 17.1 ± 0.5 | –       | –       |
| 4      | 23.0 ± 0.4       | 25.1 ± 0.5       | 19.2 ± 0.5 | 24.5 ± 0.3 | 23.1 ± 0.5 | –       |
| 5      | –                | –                | –         | –         | –       | –       |
| 6      | 20.8 ± 1.5       | 17.8 ± 1.2       | 11.9 ± 1.3 | 16.3 ± 0.5 | –       | –       |
| 7      | 12.7 ± 0.6       | 21.5 ± 1.5       | 16.9 ± 1.2 | 11.9 ± 1.3 | –       | –       |
| 9      | 22.2 ± 0.6       | 23.2 ± 1.5       | 17.2 ± 1.3 | 24.0 ± 1.3 | 16.6 ± 1.3 | –       |
| 10     | 14.2 ± 0.4       | 11.3 ± 0.7       | 12.3 ± 0.4 | 22.6 ± 1.5 | 12.1 ± 1.5 | –       |
| 12     | 18.6 ± 1.2       | 24.3 ± 0.6       | 15.8 ± 1.5 | 21.8 ± 1.3 | 20.7 ± 1.2 | –       |
| 14     | –                | –                | –         | –         | –       | –       |
| 16     | 22.8 ± 0.7       | 18.8 ± 1.4       | 18.1 ± 0.6 | 23.7 ± 0.7 | 22.9 ± 1.3 | –       |
| 17     | 21.9 ± 1.2       | 20.2 ± 1.2       | 15.5 ± 1.2 | 20.5 ± 1.3 | 16.9 ± 1.2 | 25.4 ± 0.1 |
| Amphotericin B | – | – | – | – | 23.7 ± 1.1 | – |
| Ampicillin | 23.8 ± 0.71 | 26.4 ± 0.50 | – | – | – | – |
| Gentamicin | – | – | 19.7 ± 0.6 | 24.9 ± 1.5 | – | – |

- No activity under the screening conditions; SEM: standard error mean; each value is the mean of three values.

### Biological activity

#### Antimicrobial activity

The newly prepared targets were subjected for **in-vitro** antibacterial screening against Gram-positive bacteria (Streptococcus pneumoniae and Bacillus subtilis) and Gram-negative bacteria (Pseudomonas aeruginosa and Escherichia coli). Also, these compounds were tested for their antifungal activity against Aspergillus fumigatus, and Candida albicans. The compounds’ solutions of concentrations (1 mg/mL) were evaluated against the different microorganism’s and the inhibition zone (IZ) used diameter in mm for the biologically activity (agar well diffusion method). The results are depicted in Table 1. From the screening results, we noted that compounds 3, 4, 6, 7, 9, 10, 12, 16, and 17 exhibited significant activity ranging from moderate to excellent against all tested microorganisms (Table 1). Compound 4 explored the best potential MIC values ranged from 0.49 ± 0.39 to 1.95 ± 0.23 μg/mL in comparison with that of the standard compounds, followed by 9, 16, 12 and 17 (MIC 3.90 ± 0.01–62.50 ± 0.71 μg/mL).

#### Structure–activity relationship (SAR) for antimicrobial activity

For compound 3 the presence of ethyl 2-cyano-2-(2-hydrazono)acetate moiety at 2-position of isoindoline nucleus improved antibacterial activities against all tested microorganisms (compound 3). On the other hand, combination of isoindoline nucleus with pyrazole moiety increased the antibacterial activities and antifungal activity against A. fumigatus (compound 4). However, the existence of N,N-dimethyl formimidamide substituent decreased the antibacterial and antifungal activities against the tested microorganisms but increased the antibacterial activity against E. coli (compound 10). In the series of substituted imidazol[1,2-b]pyrazol-7-yl)diazeyl(isoindoline 5–7, the presence of amino and phenyl substituents at the position-2 of imidazole ring enhanced the antibacterial activities and showed no activity against the tested fungi (compound 6 and 7). In contrast compound 5, which has oxo group at position-2, was found to be inactive against all the tested bacteria and fungi. Furthermore, the presence of CN group at
position-6 in the pyrazolo[1,5-a]-pyrimidine moiety enhanced the antibacterial and antifungal activities (compounds 9 and 12), while insertion of benzoyl group at position-6 deactivate the tested compound 14.

Anticancer activity

All synthesized compounds were screened for their anticancer activity against a human liver (HepG-2) cell line using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and vinblastine was used as a standard drug. Cytotoxic activity was depicted in Table 3. Usage of the data to draw a dose-response curve in which the concentrations of the evaluated compounds required to kill fifty percent of cell population (IC50) was decided. The results are represented in Table 3 and Figure 2 showed that compound 4 is the most potent cytotoxic derivative and at the same time is relatively equipotent in activity with the reference drug Vinblastine (IC50 = 4.22 ± 1.04, 4.63 ± 1.07 µg/mL, respectively). Promising activity was displayed with the following compounds 9 > 16 > 12 > 17, in a descending order (IC50 range 5.60 ± 1.57–9.92 ± 1.28 µg/mL). Also, compounds 3, 6 and 10 exhibited moderate anticancer activity opposite to the liver carcinoma cell line (HepG-2) (IC50 range 12.60 ± 0.13–16.72 ± 0.26 µg/mL). Moreover, compounds 14, 5, and 7 were less active among their analogues.

All compounds were subjected for cytotoxic screening against (THLE-2) normal liver cell line and results demonstrated IC50 values (µg/mL) of the synthesized derivatives ranging from 597.83 ± 0.14 to 874.31 ± 0.22, in comparison with the reference drug Vinblastine (IC50 = 2146.05 ± 0.10 µg/mL), respectively. These findings exhibited that all compounds had higher IC50 values against normal THLE-2 cells comparing with their IC50 doses against the cancer cells (Table 3).

Structure–activity relationship (SAR) for cytotoxic activity

It was noticed that there is a great similarity between SAR for antimicrobial activity and that for cytotoxic activity. Substitution at p-2 of isoindoline scaffold with the open chain ethyl 2-cyano-2-(2-hydrazono)acetate moiety in compound 3, exhibited promising antitumor activity against HepG-2 cell line. Cyclization at p-2 of isoindoline nucleus with 3-aminopyrazolone moiety via hydrazinyl linker in compound 4, afforded the highest potency among other derivatives. Substituent variation of NH2 group at p-3 of pyrazole nucleus with 3-aminopyrazolone moiety via hydrazinyl linker in compound 4, afforded the highest potency among other derivatives. Substituent variation of NH2 group at p-3 of pyrazole nucleus with 3-aminopyrazolone moiety via hydrazinyl linker in compound 4, afforded the highest potency among other derivatives.
In-vitro enzyme assay on DNA gyrase B and VEGFR-2
The antimicrobial and the cytotoxic results revealed that analog 4 exhibited the highest activity among other analogs. So, subsequent mechanistic studies were supplied through investigating the binding affinity of representative active derivative 4 to E. coli DNA gyrase B and VEGFR-2 kinases assaying their effects using suitable positive controls, Novobiocin and Staurosporine, respectively. From Table 4, it was observed that compound 4 represented a nearly equipotent IC50 value with Novobiocin as DNA gyrase B inhibitor (IC50 = 0.34 ± 0.63 and 0.28 ± 1.45 μM, respectively). On the other hand, it exhibited excellent and two folds the inhibitory activity of Staurosporine towards VEGFR-2 (IC50 = 0.09 ± 1.30 and 0.17 ± 1.02 μM, respectively).

In silico calculations of molecular properties

**Drug-Likeness parameters:**
Molecular descriptors illustrate the pharmacokinetic, pharmacodynamic and physicochemical properties of the compounds 3–17 exhibiting good oral bioavailability of these derivatives theoretically. The calculation results shown in Table 4 revealed that most of the compounds follow the Lipinski rules of the five51,52, revealing that there would not be problems with oral bioavailability of these compounds theoretically. Expected poor intestinal absorption was accompanied with molecules having TPSA values around 140Å2 or more. Thus, all compounds (except 9, 12, 14, 16 and 17) have represented a TPSA less than 140Å2, exhibiting a good permeability of the drug in the cellular plasma membrane. It has been shown that for the compound to have a reasonable probability of being well absorbed, mLogP value must be in the range of −0.4 to +544. On this basis, all the synthesized compounds were found to have mLogP values under the acceptable criteria and they are expected to have good oral absorption (Table 5). Also, compounds 9 and 12 have shown very high percentage of absorption (%ABS), that is a parameter of good bioavailability via oral administration but the rest of compounds have a reasonable probability of absorption. Molecules with more than 10 rotatable bonds may have problems with bioavailability44. All the tested compounds have 2–5 rotatable bonds and they might not have problems with bioavailability. Furthermore, all noNH values (H-bond donors) are in the range of 1–4 indicating their solubility in cellular membranes. All compounds having one or zero violation of Lipinski’s rule are expected not to have problems with bioavailability (Table 5), while those violating more than one may have problems with bioavailability53.

The toxicity risk assessment (TRA) indicators, including irritant, tumorigenic, mutagenic and reproductive effects are the tools for the risks of toxicity. This assessment proposed that compounds 5, 6, 7, 9, 12 and 14 did not show any toxicity risk profile. However, compounds 16 and 17 showed the low of mutagenic and high tumorigenicity effects, respectively. Also, compounds 3, 4 and 10 have shown the high of irritancy and low reproductive effects, respectively (Table 6). The absorption and distribution characteristics of a compound were significantly affected by its aqueous solubility. It is well known that low solubility is accompanied with bad absorption and the general aim is to be away from the poorly soluble compounds. So, there are more than 80% of the drugs on the market having solubility values greater than −4. Table 6 showed those compounds 3, 4, 5, 6 and 10 exhibiting solubility values above −4 and they are suggested to have good aqueous solubility which significantly influences their absorption and distribution characteristics. Drug-likeness with a positive value points that the derivative consists of fragments involved in most applicable drugs. The drug score merge the risk of toxicity, solubility, lipophilicity, drug-likeness and molecular weight into a single numerical value which can be applied to foretell a global value for each derivative as a potential new drug candidate54. The data shown in Table 6 indicated that all compounds have displayed

### Table 4. Inhibitory evaluation of compound 4 against DNA gyrase B and VEGFR-2 kinases.

| Comp. No | DNA gyrase B (IC50 Mean ± SEM) (μM) | VEGFR-2 (IC50 Mean ± SEM) (μM) |
|----------|----------------------------------|----------------------------------|
| 4        | 0.34 ± 0.63                      | 0.09 ± 1.30                     |
| Novobiocin | 0.28 ± 1.45                   | –                               |
| Staurosporine | –                               | 0.17 ± 1.02                   |

### Table 5. Calculated molecular properties of the synthesized compounds for assessment of the drug likeness

| Comp. No | Rule | mLogP | % ABS | TPSA | N atoms | MW (<500) | M. Vol. | % OH (<10) | % NH (<5) | % rotb. (<10) |
|----------|------|-------|-------|------|---------|----------|---------|------------|-----------|------------|
| 3        | 1    | 1.22  | 69.83 | 113.56 | 21      | 286.25   | 237.81  | 8          | 1         | 0          | 5         |
| 4        | 0.75 | 0.25  | 62.65 | 135.24 | 20      | 277.22   | 214.71  | 9          | 4         | 0          | 2         |
| 5        | 0.27 | 0.27  | 63.82 | 130.96 | 23      | 312.25   | 241.04  | 10         | 2         | 0          | 2         |
| 6        | 1.02 | 1.02  | 60.62 | 140.25 | 23      | 311.26   | 244.20  | 10         | 3         | 0          | 2         |
| 7        | 3.40 | 3.40  | 68.60 | 117.13 | 28      | 327.34   | 304.08  | 9          | 2         | 0          | 3         |
| 9        | 2.04 | 2.04  | 86.91 | 164.05 | 28      | 394.38   | 306.37  | 11         | 3         | 1          | 3         |
| 10       | 0.73 | 0.73  | 65.94 | 124.82 | 24      | 327.30   | 272.59  | 10         | 2         | 0          | 4         |
| 12       | 1.15 | 1.15  | 86.91 | 164.05 | 26      | 348.28   | 271.68  | 11         | 3         | 1          | 2         |
| 14       | 2.85 | 2.85  | 54.73 | 157.32 | 32      | 427.38   | 345.21  | 11         | 3         | 1          | 4         |
| 16       | 1.10 | 1.10  | 47.96 | 176.94 | 26      | 349.27   | 267.52  | 12         | 3         | 1          | 2         |
| 17       | 2.16 | 2.16  | 56.07 | 153.43 | 29      | 395.33   | 317.26  | 12         | 1         | 1          | 5         |
| Ampthoter-in C | −2.49 | −2.49 | 1.27  | 319.61 | 65      | 924.09   | 865.48  | 18         | 3         | 3          | 3         |
| Ampicillin     | −0.87 | −0.87 | 70.11 | 112.73 | 24      | 349.41   | 298.87  | 7          | 4         | 0          | 4         |
| Gentamycin     | −4.21 | −4.21 | 40.18 | 199.74 | 33      | 477.60   | 450.66  | 12         | 11        | 2          | 7         |
| Vinblastine    | 5.56  | 5.56  | 55.84 | 154.11 | 59      | 810.99   | 744.65  | 13         | 3         | 3          | 10        |

4Octanol-water partition coefficient, calculated by the methodology developed by Molinspiration.
5% ABS percentage of absorption.
6TPSA topological polar surface area.
7Number of non-hydrogen atoms.
8Molecular weight.
9Molecular volume.
10Number of hydrogen-bond acceptors (O and N atoms).
11Number of hydrogen-bond donors (OH and NH groups).
12Number of “Rule of five” violations.
13Number of rotatable bonds.
negative values of drug likeness in the comparable zone with that of the standard drugs. The drug score calculation of all compounds revealed positive values ranged from 0.18 to 0.45. Even those compounds with negative drug likeness have illustrated positive drug scores (Table 6). Finally, it could be observed that compounds 5 and 6 have potential as new drug candidates, but the rest of the series have drug scores from low to moderate values comparing with the reference drugs used.

Table 6. Toxicity risks, solubility, drug-likeness, and drug score of the target derivatives.

| Comp. no. | Mutagen-icity | Tumorigen-icity | Irritancy | Reproductive effect | Solubility | Drug-likeness | Drug Score |
|-----------|---------------|-----------------|-----------|---------------------|------------|---------------|------------|
| 3         | No risk       | No risk         | high risk | low risk            | –2.6       | –12.44        | 0.22       |
| 4         | No risk       | No risk         | high risk | low risk            | –2.26      | –2.18         | 0.25       |
| 5         | No risk       | No risk         | No risk   | No risk             | –3.01      | –7.17         | 0.44       |
| 6         | No risk       | No risk         | No risk   | No risk             | –2.81      | –7.51         | 0.45       |
| 7         | No risk       | No risk         | No risk   | No risk             | –5.25      | –8.23         | 0.31       |
| 9         | No risk       | No risk         | No risk   | No risk             | –5.64      | –12.59        | 0.3        |
| 10        | No risk       | No risk         | high risk | low risk            | –2.32      | –0.86         | 0.28       |
| 12        | No risk       | No risk         | No risk   | No risk             | –5.03      | –12.32        | 0.35       |
| 14        | No risk       | No risk         | No risk   | No risk             | –6.35      | –7.14         | 0.25       |
| 16        | low risk      | high risk       | No risk   | No risk             | –4.6       | –12.94        | 0.18       |
| 17        | low risk      | high risk       | No risk   | No risk             | –4.03      | –11.35        | 0.18       |
| Amphote-rin B | No risk   | No risk         | No risk   | No risk             | –5.08      | –0.14         | 0.27       |
| Ampicillin | No risk       | No risk         | No risk   | No risk             | –1.57      | 10.72         | 0.91       |
| Gentamy-cin | No risk     | No risk         | No risk   | No risk             | –1.18      | 4.88          | 0.77       |
| Vinblastine | No risk     | No risk         | No risk   | No risk             | –5.08      | 5.61          | 0.35       |

Figure: 3. A & B images show 2 D and 3 D docking view of compound 4 in the binding site of DNA gyrase (pdb code: 1KZN), hydrogen bonds are illustrated as dotted purple lines; C atoms are colored gray, N blue and O red.
Based on the kinase assessment observations and the previous literatures illustrated the important correlation between phthalimide analogs and *E. coli* topoisomerase II DNA gyrase B as antibacterial target and VEGFR-2 as anticancer core, we decided to investigate the possible interactions and binding modes of compound 4 with the active sites of those enzymes. Docking simulations were performed using the X-ray crystallographic structures for DNA gyrase B (PDB ID: 1KZN) with the natural inhibitor clorobiocin and for VEGFR-2 (pdb code: 2OH4) with the original ligand GIG. The cocrystallized ligands clorobiocin and GIG were redocked into the pocket sites of DNA gyrase B and VEGFR-2 and revealed docking score energies \(-11.4, -13.7\) kcal/mol at RMSD value (root mean square deviation) equal 9.3, 8.5, respectively. The energy is minimized for compound 4 in 3D picture, and then it saved in a molecular data base (MDB) file to be docked into the active sites of the two enzymes. It showed score energies lower than the cocrystallized ligands \(-12.3, -15.6\) kcal/mol with DNA gyrase B and VEGFR-2, respectively.

The binding map of compound 4 in the pocket of DNA gyrase B was explained through two stable hydrogen bonding interactions between the two carbonyl groups of phthalimide scaffold and the sidechains of Asn46 and Thr165 (distance: 3.12 and 2.66 Å). Furthermore, the essential amino acid Asp73 located in the motif II of N-terminal loop provided a hydrogen bond with the NH proton of pyrazolone moiety (distance: 1.64 Å) (Figure 2).

Docking study results of compound 4 inside the ATP binding site of VEGFR-2 revealed that the carbonyl group of phthalimide moiety formed H-bond acceptor with the backbone of Asp1044 oriented in the C-terminal domain (distance: 2.18 Å). Furthermore, the two amino protons of pyrazolone ring formed H-bond donors with the side chain of Glu883 located in the N-terminal lobe and

**Figure: 4.** A & B images show 2D and 3D docking view of compound 4 in the binding site of VEGFR-2 (pdb code: 2OH4), hydrogen bonds are illustrated as dotted purple lines; C atoms are colored gray, N blue and O red.

**Molecular modeling study**

Based on the kinase assessment observations and the previous literatures illustrated the important correlation between phthalimide analogs and *E. coli* topoisomerase II DNA gyrase B as antibacterial target and VEGFR-2 as anticancer core, we decided to investigate the possible interactions and binding modes of compound 4 with the active sites of those enzymes. Docking simulations were performed using the X-ray crystallographic structures for DNA gyrase B (PDB ID: 1KZN) with the natural inhibitor clorobiocin and for VEGFR-2 (pdb code: 2OH4) with the original ligand GIG. The cocrystallized ligands clorobiocin and GIG were redocked into the pocket sites of DNA gyrase B and VEGFR-2 and revealed docking score energies \(-11.4, -13.7\) kcal/mol at RMSD value (root mean square deviation) equal 9.3, 8.5, respectively. The energy is minimized for compound 4 in 3D picture, and then it saved in a molecular data base (MDB) file to be docked into the active sites of the two enzymes. It showed score energies lower than the cocrystallized ligands \(-12.3, -15.6\) kcal/mol with DNA gyrase B and VEGFR-2, respectively.

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Docking study results of compound 4 inside the ATP binding site of VEGFR-2 revealed that the carbonyl group of phthalimide moiety formed H-bond acceptor with the backbone of Asp1044 oriented in the C-terminal domain (distance: 2.18 Å). Furthermore, the two amino protons of pyrazolone ring formed H-bond donors with the side chain of Glu883 located in the N-terminal lobe and

**Figure: 4.** A & B images show 2D and 3D docking view of compound 4 in the binding site of VEGFR-2 (pdb code: 2OH4), hydrogen bonds are illustrated as dotted purple lines; C atoms are colored gray, N blue and O red.
the backbone of Phe1045 inserted in the C-terminal domain (distance: 1.97, 1.93 Å, respectively) (Figure 3).

Finally, the docking analysis was agreed with the previous antimicrobial, anticancer and enzyme inhibitory activities and could explain how the phthalimide moiety played a pivotal role in stability in the ATP binding sites of both enzymes through its carbonyl groups. Also, the amino group of pyrazolone ring contributed considerably to the strength of binding interactions.

Conclusion
A new imidazopyrazole, pyrazolopyrimidine and pyrazolo-1,2,4-triazine derivatives containing phthalimide moiety were prepared and in vitro antimicrobial and anticancer were reported. Compound 4 was the most active compound against Gram positive bacteria (S. pneumoniae and B. subtilis), Gram negative bacteria (P. aeruginosa and E. coli) and fungi (A. fumigatus). Also, compound 4 was the most potent compound in cytotoxic assay against hepatic cancer cell line (HepG-2) in comparison with the standard drug vinblastine. Drug-likeness and Toxicity risk parameters of the newly synthesized compounds were calculated using in silico studies. The promising results motivated us to perform enzyme assay and docking simulations to gain insight into the plausible mechanism of antibacterial and cytotoxic activities of target compound 4 as DNA gyrase and VEGFR-2 inhibitors. The obtained findings may open up new possibilities for developing a new class of phthalimide drugs with antimicrobial and cytotoxic activity.

Disclosure statement
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