Design, synthesis, and biological evaluation of novel bioactive thalidomide analogs as anticancer immunomodulatory agents†

Anas Ramadan Kotb,a Dina A. Bakhotmah,⁎b Abdallah E. Abdallah,⁬a Hazem Elkady,⁢a Mohammed S. Taghour,a Ibrahim. H. Eissa ⁣⁢a and Mohamed Ayman El-Zahabi∗a

Cancer is still a dangerous disease with a high mortality rate all over the world. In our attempt to develop potential anticancer candidates, new quinazoline and phthalazine based compounds were designed and synthesized. The new derivatives were built in line with the pharmacophoric features of thalidomide. The new derivatives as well as thalidomide were examined against three cancer cell lines, namely, hepatocellular carcinoma (HepG-2), breast cancer (MCF-7) and prostate cancer (PC3). Then the effects on the expression levels of caspase-8, VEGF, NF-κB P65, and TNF-α in HepG-2 cells were evaluated. The biological data revealed the high importance of phthalazine based compounds (24a–c), which were far better than thalidomide with regard to the antiproliferative activity. 24b showed IC50 of 2.51, 5.80 and 4.11 μg mL⁻¹ compared to 11.26, 14.58, and 16.87 μg mL⁻¹ for thalidomide against the three cell lines respectively. 24b raised caspase-8 level by about 7 folds, compared to 8 folds reported for thalidomide. Also, VEGF level in HepG-2 cells treated with 24b was 185.3 pg mL⁻¹, compared to 432.5 pg mL⁻¹ in control cells. Furthermore, the immunomodulatory properties were proven to 24b, which reduced TNF-α level by approximately half. At the same time, NF-κB P65 level in HepG-2 cells treated with 24b was 76.5 pg mL⁻¹ compared to 278.1 and 110.5 pg mL⁻¹ measured for control cells and thalidomide treated HepG-2 cells respectively. Moreover, an in vitro viability study against Vero non-cancerous cell line was investigated and the results reflected a high safety profile of all tested compounds. This work suggests 24b as a promising lead compound for development of new immunomodulatory anticancer agents.

In our journey to find a suitable lead, thalidomide emerged as a lead with many significant criteria. Aside from teratogenicity, thalidomide was found to exhibit few side effects.10 It was modified to give what is known as immunomodulatory drugs (IMiDs), such as lenalidomide and pomalidomide, that were approved for cancer treatment.11–13 The significant anticancer effects of thalidomide and the IMiDs have been proven to involve different targets and several mechanisms, which include immunomodulatory action. Vascular endothelial growth factor (VEGF) and hence angiogenesis were reported to be inhibited by these drugs.14 Also, the apoptosis rate and cell cycle arrest were increased along with the elevation of caspase-8 levels.14 Furthermore, the immunomodulatory effects that include co-stimulation of T-cells, inhibition of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1, IL-12. In addition to nuclear factor kappa B (NFκB) inhibition.14 On the other hand, the levels of the anti-inflammatory cytokines were elevated. The elevated cytokines include interferon-gamma (IFN-γ) and IL-10, IL-2.11,15–19 Thalidomide’s immunomodulatory properties account for its efficacy in various autoimmune and inflammatory diseases.20,21 Based on these characteristics, we suggest that thalidomide

1. Introduction

In the last few decades, there has been a tremendous amount of effort and progress in cancer treatment. However, cancer is still a disease with an increasingly high mortality rate all over the world.1 Many challenges have been reported clinically, including resistance of cancer to the used drugs, which leads to failure of the treatment.2–4 In addition to that, the toxicity of many anticancer drugs is not limited to cancer cells.5 As a result, severe and serious toxic effects were reported5,7 as well as difficulties in optimizing the drug regimen.6 These two main problems were taken into account in this study which aimed to develop anticancer candidates with potential low resistance rate and few side effects.8

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modification may afford new effective and safe candidates with a low resistance rate.

Thalidomide structural modifications have resulted in many effective anticancer analogs such as lenalidomide, pomalidomide, and avadomide (CC-122). Thalidomide was approved for treatment of acute ENL in 1998 and for treatment of multiple myeloma (MM) in 2006. Lenalidomide was found to be a more potent TNF-α inhibitor than thalidomide. In 2006, it received FDA approval for treatment of MM. Pomalidomide showed more significant results than lenalidomide in both inhibition of TNF-α and stimulation of IL-2. In 2013, it was approved by FDA for treatment of MM. Avadomide exhibited potent antiproliferative, anti-angiogenic and immunomodulatory activities. It was proven to be effective in treatment of MM, diffuse large B-cell lymphoma (DLBCL) and solid tumors.

The structural modification of thalidomide that involved the replacement of the phthalimido moiety by quinazoline nucleus afforded avadomide. Quinazoline containing candidates revealed potent inhibition to NF-κB, TNF-α, IL-6, and IL1β along with induction of apoptosis and elevation of caspase levels. The compounds based on phthalazine, a bio-isostere of quinazoline, showed similar properties which included TNF-α and NF-κB inhibition, antiproliferative, apoptosis induction and antiangiogenic effect.

1.1. Rationale of molecular design

It is clear that thalidomide has many targets involved in the expression of its high efficacy in cancer, autoimmune and inflammatory diseases. As a result, analyzing the binding interactions to one or two targets does not provide an explanation for all the biological effects reported for thalidomide. But analyzing the chemical structure of thalidomide may be a better way to design new candidates with similar properties. Hence, the ligand-based design approach has been followed in this study. The molecular structure of thalidomide as a lead compound can be analyzed into four main regions as illustrated in Fig. 1. Four pharmacophoric features are identified as follows: (1) a flat hetero aromatic ring, (2) a spacer, (3) a hydrogen bond forming region, (4) a hydrophobic moiety.

Herein, we carried out modifications on the analyzed four regions. Firstly, the phthalimide moiety was replaced with two other bio-isostere heteroaromatic nuclei; quinazoline and phthalazine. It was reported that quinazoline was a significant alternative to phthalimide and afforded the highly effective avadomide molecule. Secondly, it is noticed that the rotation of the sigma bond between glutarimide moiety and phthalimide nucleus of thalidomide is almost restricted. We aimed to increase the flexibility by having one or two NH groups which will allow more flexibility of the attached planar
benzenesulfonyl, the chloro-fluoranilide moieties or the puckered glutarimide ring or ring open modification of the semicarbazide moiety. Consequently, such possible variations may result in biological variations due to possible variations of the way of binding to the target binding sites as shown in Fig. 1. Thirdly, the hydrogen bond forming group was designed to include imide, urea, sulfamoyl, and even hydrogen bond forming halogens e.g., fluoro and chloro. Finally, aliphatic and aromatic groups were constructed to examine which would be the most effective as a hydrophobic moiety in terms of thalidomide activities.

2. Results and discussion

2.1. Chemistry

For synthesis of the target compounds, reactions sequence is clarified in Schemes 1–3. Initially, quinazoline-2,4(1H,3H)-dione 3 was prepared by fusion of anthranilic acid 1 with urea 2 according to the reported methods. Next, heating of 3 with phosphorus oxychloride in the presence of Et3N for 7 h afforded the dichloro derivative 4. The produced 2,4-dichloroquinazoline 4 was refluxed with 3-chloro-4-fluoroaniline 5 in isopropyl alcohol in the presence of two mole equivalents of Et3N to produce the final target compound 6. Refluxing two-mole equivalents of methyl and ethyl amine with compound 6 in acetonitrile gave compounds 7a,b respectively (Scheme 1). The structures of compounds 6 and 7a,b were verified by elemental analyses and spectral data. The IR spectra of these compounds revealed the presence of NH absorption bands at a range of 3380–3277 cm⁻¹. Moreover, the ¹H NMR spectra of compound 6 showed peak at 10.26 ppm due to NH. On the other hand, the ¹H NMR spectra of compounds 7a,b showed deshielded peaks at a range of 8.30 to 9.5 ppm corresponding to the two NHs. Additionally, the aliphatic protons appeared at a range of 1.02 to 3.48 ppm.

In Scheme 2, the starting materials 9a,b were reported to be prepared by fusion of urea with 5-chloroaanthranilic acid 8a or 6-chloroaanthranilic acid 8b, respectively. Chlorination of the produced quinazoline-2,4-dione derivatives 9a,b was achieved by refluxing in POCl₃ in the presence of N,N-dimethyl aniline (DMA) following the reported procedure. The target compounds 11a,b were finalized by refluxing 2,4,5-trichloro-1,2,3,4-tetrahydroquinazoline 10a and/or 2,4,6-trichloro-1,2,3,4-tetrahydroquinazoline 10b with 3-chloro-4-fluoroaniline 5 in isopropyl alcohol in the presence of Et3N. IR spectra revealed the presence of NH absorption bands at 3419 and 3381 cm⁻¹. On the other hand, the ¹H NMR spectra of compounds 11a,b showed deshielded peaks for the NH protons at a range of 10.31 to 9.87 ppm. In the meantime, compounds 13a,b were furnished by refluxing the appropriate 2,4-dichloroquinazoline derivatives 10a,b with 3-aminopiperidine-2,6-dione HCl 12 under basic conditions. Also, under the same condition reactions, compounds 15a,b was produced by reaction of 10a,b with semicarbazide HCl 14. Treatment of 2,4,5-trichloroquinazoline 10a with excess hydrazine hydrate afforded 2,5-dichloro-4-hydrazinylquinazoline 17. Finally, synthesis of compounds 19a,b was attained in good yields by dropwise addition of the appropriate benzenesulfonyl chloride 18a,b to a mixture of 2,5-dichloro-4-hydrazinylquinazoline 17 and Et3N in DMF in ice salt bath. The IR spectra of these compounds were characterized by appearance of characteristic bands of the two NH groups at 3397–3306 cm⁻¹ and very characteristic bands of SO₂ group at a range of 1166–1163 cm⁻¹. On the other hand, the ¹H NMR

Scheme 1  General procedure for preparation of target compounds 6 and 7a,b.
spectrum of 19a showed peaks at 10.04 and 7.82 ppm due to sulfonhydrazide moiety while compound 19b showed peaks at 10.24, 7.97 ppm.

2,3-Dihydrophthalazine-1,4-dione 21 was prepared by condensation of equimolar amounts of phthalic anhydride 20 and hydrazine hydrate 16 in absolute ethanol, according to a reported procedure as illustrated in Scheme 3. Refluxing of 21 in POCl3 afforded 1,4-dichlorophthalazine 22, which was then treated with an excess of hydrazine hydrate 16 to give 1-chloro-4-hydrazinylphthalazine 23 as shown in Scheme 3. Finally, the target compounds 24a–c were obtained by addition of an appropriate benzenesulfonyl chloride 18a–c dropwise to a cooled solution of 23 in DMF. The 1H NMR spectra of 24a–c showed peaks at about 11.75 and 9.44 ppm due to sulfonhydrazide moiety.

2.2. Biological testing

2.2.1. In vitro anti-proliferative activity. The new candidates were evaluated against three cancer cell lines, namely: hepatocellular carcinoma (HepG-2), breast cancer (MCF-7) and prostate cancer (PC3). The concentrations that caused 50% growth inhibition (IC50) were calculated. Thalidomide was used as a positive reference. The data obtained are presented in Table 1.

It is noticeable that the 24a–c compounds, which are based on the phthalazine core carrying the sulfonhydrazide moiety, showed the best antiproliferative results. Such compounds are, in fact, significantly better than thalidomide versus the selected cancer cell lines. The most significant candidate was 24b, which showed IC50 of 2.51, 5.80 and 4.11 µg mL⁻¹ compared to 11.26, 14.58, and 16.87 µg mL⁻¹ for thalidomide against the three cell
Three independent experiments were performed for each concentration. It was found clear that HepG-2 was the most sensitive cancer cell line for the most promising candidates (24a-c). Accordingly, HepG-2 cell line was selected for further evaluation. Two compounds were selected for such evaluation; phthalazine based 24b (the most effective candidate) and 7b (the most promising quinazoline based derivative).

2.2.2. Structure activity relationship. Observing the results of biological testing (in vitro anti-proliferative activity), we could determine valuable data about the structure–activity relationships.

Regarding quinazoline containing derivatives, comparing the cytotoxic activity of compounds 11b, 13b, and 15b (incorporating 2,6-dichloroquinazoline moiety) with compounds 11a, 13a, and 15a (incorporating 2,5-dichloroquinazoline moiety) indicated that 2,6-dichloroquinazoline moiety is more advantageous than 2,5-dichloroquinazoline moiety for cytotoxic activity against all cell lines. Moreover, comparing the activity of compounds 15a and 15b having semicarbazide moiety with compounds 13a and 13b having glutarimide moiety indicated that semicarbazide moieties are more beneficial for activity. Regarding 2-chloroquinazoline derivatives 6, 7a,b, it was noticed that replacement of chlorine atom at position-2 with methyleamine increased the cytotoxic activity against all cell lines. However, replacement of methyleamine with ethylamine increased the cytotoxic activity against all cell lines as well. With regard to phthalazine containing derivatives, comparing the activity of compound 24a (in which 4-methylbenzenesulfonylhydrazide moiety is incorporated) and compound 24b (in which 4-fluorobenzenesulfonylhydrazide moiety is incorporated) with compound 24a (in which benzenesulfonylhydrazide moiety is incorporated), revealed that substituted moieties are more potent than unsubstituted one. In the meantime, comparing the activity of compound 24c (incorporating 4-fluorobenzenesulfonylhydrazide moiety) with

Table 1  Anti-proliferative activities of the target compounds against HepG-2, PC3 and MCF-7 cell lines

| Comp | HepG-2 (μg mL⁻¹) | MCF-7 (μg mL⁻¹) | PC3 (μg mL⁻¹) |
|------|-----------------|----------------|---------------|
| 6    | 74.81 ± 4.1     | 81.52 ± 4.2    | 84.96 ± 4.7   |
| 7a   | 40.50 ± 2.8     | 28.13 ± 2.1    | 47.20 ± 2.8   |
| 7b   | 11.68 ± 0.8     | 9.45 ± 0.9     | 15.48 ± 1.3   |
| 11a  | 77.24 ± 4.2     | 86.14 ± 4.4    | 91.26 ± 4.9   |
| 11b  | 38.74 ± 2.8     | 45.91 ± 3.0    | 51.20 ± 3.5   |
| 13a  | 46.32 ± 3.1     | 35.29 ± 2.4    | 54.31 ± 3.0   |
| 13b  | 59.01 ± 3.3     | 29.33 ± 2.3    | 38.36 ± 3.1   |
| 15a  | 24.51 ± 2.0     | 17.33 ± 1.3    | 26.08 ± 2.0   |
| 15b  | 16.18 ± 1.4     | 9.87 ± 0.9     | 13.79 ± 1.2   |
| 19a  | 19.07 ± 1.5     | 12.76 ± 1.0    | 21.48 ± 1.6   |
| 19b  | 28.92 ± 2.2     | 20.48 ± 1.5    | 32.71 ± 2.3   |
| 24a  | 3.87 ± 0.2      | 6.07 ± 0.7     | 8.63 ± 0.6    |
| 24b  | 2.51 ± 0.1      | 5.80 ± 0.4     | 4.11 ± 0.3    |
| 24c  | 7.96 ± 0.5      | 8.12 ± 0.7     | 14.92 ± 1.1   |
| Thalidomide | 11.26 ± 0.54 | 14.58 ± 0.57  | 16.87 ± 0.7   |

* Three independent experiments were performed for each concentration.

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compound 24b (incorporating 4-methylbenzenesulfonyldrazide moiety), exhibited that the electron withdrawing group is more preferred biologically than electron donating one (Fig. 2).

2.2.3. In vitro protein expression assay. Compounds 24b and 7b, along with thalidomide as a positive reference, were tested in HepG-2 cells for their effects on the expression levels of caspase-8 and VEGF.

2.2.3.1. The effect on the expression level of caspase-8. It was found that compound 24b was able to increase caspase-8 level in HepG-2 cells by about 7 folds, compared to 8 folds reported for thalidomide. While 7b revealed a threefold increase in caspase-8 level as shown in Table 2.

2.2.3.2. The effect on the expression level of vascular endothelial growth factor (VEGF). The data presented in Table 2 show that compound 24b reduced the level of VEGF to less than half of that of control cells. HepG-2 cells treated with 24b revealed VEGF level of 185.3 pg mL\(^{-1}\), compared to 432.5 pg mL\(^{-1}\) calculated for control cells. Additionally, 7b reduced VEGF level in HepG-2 cells by about half (218.5 pg mL\(^{-1}\)). On the other hand, VEGF level in HepG-2 cells treated with thalidomide was found to be 53.1 pg mL\(^{-1}\).

2.2.4. In vitro immunomodulatory assay. The effects of 24b and 7b, as well as thalidomide, on the expression levels of NF-\(\kappa\)B P65 and TNF-\(\alpha\) in HepG-2 were studied further.

2.2.4.1. The effect on NF-\(\kappa\)B P65 expression level. The obtained data indicated that the impact of 24b on NF-\(\kappa\)B P65 was much better than thalidomide. It can be seen that NF-\(\kappa\)B P65 level in HepG-2 cells treated with 24b was 76.5 pg mL\(^{-1}\) compared to 110.5 pg mL\(^{-1}\) measured for control HepG-2 cells and thalidomide treated HepG-2 cells respectively. At the same time compound 7b showed a result that was almost comparable to that of thalidomide (Table 2).

2.2.4.2. The effect on TNF-\(\alpha\) expression level. The data presented in Table 2 shows that both 24b and 7b reduced TNF-\(\alpha\) levels in HepG-2 cells by about half. TNF-\(\alpha\) levels were reduced from 162.5 pg mL\(^{-1}\) in control cells to 82.5 and 79.3 pg mL\(^{-1}\) under the effects of 24b and 7b respectively. On the other hand, the level in cells treated with thalidomide was 53.1 pg mL\(^{-1}\).

2.2.5. Cytotoxicity against Vero cells and selectivity index. The most promising candidates (7b and 24b) were investigated for their safety patterns against normal cell line (Vero cells). Using MTT assay, compounds 7b and 24b showed IC\(_{50}\) values of 163.61 and 161.12 pg mL\(^{-1}\). Such values were very high in comparison to the corresponding values on cancer cell lines, which reflect the high in vitro safety profile of the tested members towards non-cancerous cell line (Table 3).

The selectivity index (SI) of a particular compound is the ratio of its toxic concentration against the effective concentration (SI = IC\(_{50}\) on non-cancer cell/IC\(_{50}\) on cancer cell).\(^4\) From the results presented in Table 3, the tested compounds showed very high SI against the cancer cell lines. Interestingly, compound 24b showed the highest SI values of 65.18 and 39.80 against the HepG2, and PC3 cell lines, respectively. Compound

Table 2  The effect of the selected candidates and thalidomide on the levels of caspase-8, VEGF, NF\(\kappa\)B and TNF-\(\alpha\)

| Comp. no. | CASPASE-8 (ng mL\(^{-1}\)) | VEGF (pg mL\(^{-1}\)) | NF\(\kappa\)B P65 (pg mL\(^{-1}\)) | TNF-\(\alpha\) (pg mL\(^{-1}\)) |
|-----------|----------------------------|----------------------|---------------------|-------------------|
| 24b       | 6.9                        | 185.3                | 76.5                | 82.5              |
| 7b        | 3.2                        | 218.5                | 113.7               | 79.3              |
| Control   | 1.08                       | 432.5                | 278.1               | 162.5             |
| Thalidomide | 8.3                         | 153.2                | 110.5               | 53.1              |
7b showed SI values of 13.78, 17.04, and 10.40 against the HepG2, MCF-7, and PC3 cell lines, respectively.

2.3. In silico investigation

2.3.1. Lipinski’s rule of five and Veber’s rule. According to Veber’s and Lipinski’s rule of five, an in silico computational analysis of the tested candidates was done to ascertain their physicochemical properties. The results presented in Table 4 showed that the tested compounds showed no contravention of Lipinski’s and Veber’s rules and hence display a drug-like molecular nature. In details, the partition coefficient (logP) values were less than 5 except compounds 6, 7b, and 11a, and 13b. Molecular weight (M_{wt}) values of the tested members were less than 500. In addition, number of H-bond donors (HBD) and number of H-bond acceptors (HBA) of these compounds are within the accepted values of less than 5 and 10, respectively. Regarding Veber’s rule, the number of rotatable bonds and topological polar surface area (TPSA) of such candidates are within the acceptable values of less than 10 and 140 Å^2, respectively. These findings conclude that all the tested compounds meet the criteria of Lipinski and Veber’s rules and displayed acceptable physicochemical properties.

2.3.2. ADMET profiling study. Utilizing Discovery Studio 4.0,^{39–41} the ADMET characteristics of the examined compounds were evaluated (Table 5 and Fig. 3). As a standard drug, thalidomide was used.

Among the tested compounds, 11b, 13a, 15a, 15b, and 24c are exhibited low blood–brain barrier (BBB) penetration levels. As a result, it was anticipated that these compounds would not have CNS adverse effects. Regarding aqueous solubility parameters, all compounds showed low to very low levels of aqueous solubility. Next, the intestinal absorption levels of all compounds look like in the good and moderate range. It is interesting to note that compounds 13a,b, 15a,b, 19a,b, and 24a–c were predicted to not inhibit CYP2D6. As a result, it might

| Comp | BBB level | Solubility | Absorption | CYP2D6 inhibition | Plasma protein binding |
|------|-----------|------------|------------|-------------------|-----------------------|
| 6    | 0         | 1          | 0          | True              | True                  |
| 7a   | 1         | 1          | 0          | True              | True                  |
| 7b   | 1         | 1          | 0          | True              | True                  |
| 11a  | 0         | 1          | 1          | True              | True                  |
| 11b  | 3         | 1          | 1          | False             | True                  |
| 13a  | 3         | 1          | 0          | False             | True                  |
| 13b  | 0         | 1          | 0          | False             | True                  |
| 15a  | 3         | 2          | 0          | False             | True                  |
| 15b  | 3         | 2          | 0          | False             | True                  |
| 19a  | 2         | 2          | 0          | False             | True                  |
| 19b  | 2         | 2          | 0          | False             | True                  |
| 24a  | 2         | 2          | 0          | False             | True                  |
| 24b  | 2         | 2          | 0          | False             | True                  |
| 24c  | 3         | 2          | 0          | False             | True                  |
| Thalidomide | 3 | 3 | 0 | False | False |

Table 3 | IC_{50} values of the tested compounds against Vero cell line and their selectivity index (SI) against different cancer cell lines

| Comp | Cytotoxicity against Vero (IC_{50} μg mL^{-1}) | Selectivity index (SI) | HepG-2^a | MCF-7^b | PC3^c |
|------|---------------------------------------------|------------------------|-----------|-----------|-------|
| 7b   | 163.61 ± 1.12                               | 13.78                  | 17.04     | 10.40     |
| 24b  | 161.12 ± 1.42                               | 65.18                  | 8.59      | 39.80     |

^a SI = cytotoxicity against Vero cells/cytotoxicity against HepG-2 cell line. ^b SI = cytotoxicity against Vero cells/cytotoxicity against MCF-7 cell line. ^c SI = cytotoxicity against Vero cells/cytotoxicity against PC3 cell line.

Table 4 | Physicochemical properties of the tested members

| Comp | Lipinski rules | Veber’s rules |
|------|----------------|---------------|
|      | HBD | HBA | M_{wt} | logP | Rotatable bonds | TPSA |
| 6    | 1   | 3   | 308.138 | 5.202 | 2 | 37.81 |
| 7a   | 2   | 4   | 302.734 | 4.677 | 3 | 49.84 |
| 7b   | 2   | 4   | 316.761 | 5.025 | 4 | 49.84 |
| 11a  | 1   | 3   | 342.583 | 5.867 | 2 | 37.81 |
| 11b  | 2   | 5   | 325.15  | 2.49  | 2 | 83.97 |
| 13a  | 3   | 4   | 272.091 | 2.379 | 2 | 92.93 |
| 13b  | 1   | 3   | 342.583 | 5.867 | 2 | 37.81 |
| 15a  | 2   | 5   | 325.15  | 2.49  | 2 | 83.97 |
| 15b  | 3   | 4   | 272.091 | 2.379 | 2 | 92.93 |
| 19a  | 2   | 5   | 383.252 | 4.443 | 4 | 92.36 |
| 19b  | 2   | 5   | 387.216 | 4.162 | 4 | 92.36 |
| 24a  | 2   | 5   | 348.807 | 3.529 | 4 | 92.36 |
| 24b  | 2   | 5   | 352.771 | 3.248 | 4 | 92.36 |
| 24c  | 2   | 5   | 334.781 | 3.043 | 4 | 92.36 |
| Thalidomide | 1 | 4 | 258.229 | 0.097 | 1 | 83.55 |

Table 5 | ADMET parameters for the tested compounds and thalidomide

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be successfully metabolized and eliminated. In terms of plasma protein binding, compounds 15a, 19a,b behave similarly to thalidomide in binding in a range of less than 90%. The remainder of compounds, however, was anticipated to bind plasma protein over 90%.

2.3.3. *In silico* toxicity studies. According to the toxicity models created using the Discovery studio software,51,52 nine toxicity parameters were estimated computationally. Thalidomide was used as a reference (Table 6). For FDA rodent carcinogenicity (rat female) model, all compounds were predicted to

![Fig. 3 Computational prediction of ADMET parameters.](image)

### Table 6 *In silico* toxicity studies of the tested compounds and thalidomide

| Comp | FDA rodent carcinogenicity (rat-female) | Carcinogenic potency | Ames mutagenicity | Rat maximum tolerated dose (feed) | Rat Oral LD₅₀ | Rat chronic LOAEL | Skin irritancy | Ocular irritancy | DTP |
|------|----------------------------------------|----------------------|------------------|----------------------------------|--------------|------------------|----------------|----------------|----------------|
| 6    | Non-carcinogen                         | 18.598               | Mutagen          | 0.106                            | 0.023        | 0.021            | Non-irritant   | Mild            | Non-toxic      |
| 7a   | Non-carcinogen                         | 12.040               | Mutagen          | 0.240                            | 0.156        | 0.022            | Non-irritant   | Mild            | Non-toxic      |
| 7b   | Non-carcinogen                         | 35.737               | Non-mutagen      | 0.299                            | 0.115        | 0.020            | Non-irritant   | Mild            | Non-toxic      |
| 11a  | Non-carcinogen                         | 15.970               | Non-mutagen      | 0.088                            | 0.019        | 0.013            | Non-irritant   | Mild            | Non-toxic      |
| 11b  | Non-carcinogen                         | 0.679                | Non-mutagen      | 0.146                            | 0.169        | 0.037            | Non-irritant   | Moderate        | Non-toxic      |
| 13a  | Non-carcinogen                         | 11.211               | Non-mutagen      | 0.282                            | 0.064        | 0.038            | Non-irritant   | Mild            | Non-toxic      |
| 13b  | Non-carcinogen                         | 6.050                | Non-mutagen      | 0.088                            | 0.008        | 0.010            | Non-irritant   | Moderate        | Non-toxic      |
| 15a  | Non-carcinogen                         | 0.257                | Non-mutagen      | 0.146                            | 0.119        | 0.034            | Non-irritant   | Moderate        | Non-toxic      |
| 15b  | Non-carcinogen                         | 29.594               | Mutagen          | 0.282                            | 0.091        | 0.041            | Non-irritant   | Mild            | Non-toxic      |
| 19a  | Non-carcinogen                         | 10.189               | Non-mutagen      | 0.174                            | 0.924        | 0.041            | Non-irritant   | Mild            | Non-toxic      |
| 19b  | Non-carcinogen                         | 12.243               | Non-mutagen      | 0.185                            | 0.139        | 0.038            | Non-irritant   | Mild            | Toxic          |
| 24a  | Non-carcinogen                         | 12.655               | Non-mutagen      | 0.204                            | 3.675        | 0.097            | Non-irritant   | Mild            | Non-toxic      |
| 24b  | Non-carcinogen                         | 15.153               | Non-mutagen      | 0.217                            | 0.566        | 0.087            | Non-irritant   | Mild            | Non-toxic      |
| 24c  | Non-carcinogen                         | 38.125               | Non-mutagen      | 0.246                            | 1.043        | 0.105            | Non-irritant   | Mild            | Non-toxic      |
| Thalidomide | Carcinogen             | 26.375               | Non-mutagen      | 0.047                            | 0.835        | 0.133            | Non-irritant   | None            | Non-toxic      |

*a* Unit: mg per kg body weight per day. *b* Unit: g per kg body weight.
be non-carcinogenic. For carcinogenic potency TD50 rat model, compounds 7b, 15b, and 24c showed TD50 values of 35.737, 29.594, and 38.125 mg per kg body weight, which are higher than thalidomide (26.375). For Ames mutagenicity model, all compounds except 6, 7a, and 15b were non-mutagen. Regarding rat maximum tolerated dose model, all compounds demonstrated values higher than that of thalidomide (0.047 mg per kg body weight). Additionally, all compounds except 19b were estimated to be non-toxic against developmental toxicity potential model. For rat oral LD50 and model, all compounds except 19a (0.924 mg per kg body weight), 24a (3.675 mg per kg body weight), and 24c (1.043 mg per kg body weight) revealed oral LD50 values lower than that of thalidomide (0.835 g per kg body weight). For rat chronic LOAEL model, the tested compounds displayed LOAEL values ranging from 0.010 to 0.105 g per kg body weight. These values are lower than thalidomide (0.133 g per kg body weight). These values are lower than thalidomide (0.835 g per kg body weight). These values are lower than thalidomide (0.835 g per kg body weight). These values are lower than thalidomide (0.835 g per kg body weight).

3. Conclusion

Fourteen new compounds, based on quinazoline and phthalazine, were designed and synthesized according to the pharmacophoric features of thalidomide in order to get potential immunomodulatory anticancer agents. N-phthalazinylbenzenesulfonylhydrazide (24a–e) emerged as the most potent derivatives in terms of antiproliferative activity. Among which, 24b was the most significant, showing IC50 of 2.51, 5.80 and 4.11 μg mL−1 compared to 11.26, 14.58, and 16.87 μg mL−1 for thalidomide against HepG-2, MCF-7, and PC3 cell lines respectively. Furthermore, it exhibited immunomodulatory properties, including considerable reduction of the expression levels of TNF-α and NF-κB P65 in HepG-2 cells. Additionally, other biological activities of thalidomide were proven to show significant activity on many targets in a manner similar to thalidomide, and hence it should be considered for further evaluation and modification in order to obtain new potential immunomodulatory anticancer agents.

4. Experimental

4.1. Chemistry

All the reagents, chemicals, and apparatus were described in ESI.† The targeted compounds were furnished following the reported methods.

4.1.1. General procedure for synthesis of the target compound 6. To a solution of 2,4-dichloroquinazoline 4 (1.00 g, 5.02 mmol) and Et3N (0.87 mL, 6.03 mmol) in isopropanol, 3-chloro-4-fluoroaniline 5 (73) (0.8 g, 5.53 mmol) was added. The reaction mixture was refluxed for 4 h. After the reaction was complete (monitored by TLC), the reaction mixture was cooled to r.t. and poured portion wise to crushed ice while stirring. The obtained precipitate was collected by filtration, washed with water, dried and crystallized from ethanol to furnish the 4-aminoquinazoline derivative 6.

4.1.2. General procedure for synthesis of the target compounds 7a,b. To a suspension of 2-chloro-N-[3-chloro-4-fluorophenyl] quinazolin-4-amine (6) (0.25 g, 0.81 mmol) and Et3N (0.14 mL, 0.97 mmol) in acetonitrile, an appropriate amine was added. The reaction mixture was refluxed for 4 h. The reaction mixture was cooled to r.t. and poured portion wise to crushed ice while stirring. The obtained precipitate was collected by filtration, washed, dried and crystallized from ethanol to afford the corresponding 2,4-quinazolidinediamine derivatives (7a,b), respectively.

4.1.2.1. N2-(3-Chloro-4-fluorophenyl)-N′-methylquinazoline-2,4-diamine 7a.

Solid (yield, 85.50%); m.p. = 264–266 °C. IR (KBr, cm−1): 3371, 3277 (2NH), 3125 (CH aromatic), 2954 (CH aliphatic); 1H NMR (DMSO-d6) δ ppm: 9.48 (s, 1H, NH), 8.30 (s, 1H, NH), 8.25 (s, 1H, Ar–H), 7.93 (dd, J = 8.6, 4.2 Hz, 1H, Ar–H), 7.58 (dd, J = 8.2, 2.6 Hz, 1H, Ar–H), 7.38 (m, 2H, Ar–H), 7.15 (d, J = 7.6 Hz, 1H, Ar–H), 6.83 (m, 1H, Ar–H), 2.87 (d, J = 8.2 Hz, 3H, CH3); anal. calcd for C14H12ClF2N2O: C, 59.51; H, 4.00; N, 18.51 (18.32).

4.1.2.2. N2-(3-Chloro-4-fluorophenyl)-N′-ethylquinazoline-2,4-diamine 7b.

Solid (yield, 85.60%); m.p. = 270–272 °C. IR (KBr, cm−1): 3374, 3217 (2NH), 2979 (CH aliphatic); 1H NMR (DMSO-d6) δ ppm:
4.1.3. General procedure for synthesis of the target compounds 11a,b. 3-Chloro-4-fluoroaniline 5 (0.19 g, 1.28 mmol) was added to a mixture of a 2,4,5-trichloroquinazoline 10a or 2,4,6-trichloroquinazoline 10b (0.25 g, 1.07 mmol) and Et3N (0.19 mL, 1.28 mmol) in isopropanol. The reaction mixture was stirred at r.t. for 8 h. The formed solid was collected by filtration, washed and crystallized from ethanol to give the corresponding 4-aminquinazoline derivatives (11a,b), respectively.

4.1.3.1. 2,5-Dichloro-N-(3-chloro-4-fluorophenyl)quinazolin-4-amine 11a.

Solid (yield, 81.78%); m.p. = 132–135 ºC. IR (KBr, cm⁻¹): 3381 (1NH), 3059 (CH aromatic); ¹H NMR (DMSO-d₆) δ ppm: 9.87 (s, 1H, NH), 8.00 (s, 1H, Ar-H), 7.86 (d, J = 8.0 Hz, 1H, Ar-H), 7.73 (m, 3H, Ar-H), 7.52 (d, J = 6.8, 2.6 Hz, 1H, Ar-H); ¹³C NMR (101 MHz, DMSO-d₆) δ 158.73, 156.19, 153.85, 134.56, 129.74, 129.08, 127.23, 125.62, 124.50, 124.43, 117.35, 111.13, 111.97; mass (m/z): 343 (M⁻+ 1), 342 (M⁻), and 157 (100%, base peak). Anal. calc. for C₃₆H₂₆Cl₂N₄O²: C, 51.04; H, 2.62; N, 12.27 (12.49).

4.1.3.2. 2,6-Dichloro-N-(3-chloro-4-fluorophenyl)quinazolin-4-amine 11b.

Solid (yield, 79.06%); m.p. = 145–146 ºC. IR (KBr, cm⁻¹): 3419 (1NH), 3065 (CH aromatic); ¹H NMR (DMSO-d₆) δ ppm: 10.31 (s, 1H, NH), 8.70 (s, 1H, Ar-H), 8.09 (s, 1H, Ar-H), 7.94 (d, J = 8.1 Hz, 1H, Ar-H), 7.79 (m, 2H, Ar-H), 7.52 (d, J = 6.8, 2.6 Hz, 1H, Ar-H); ¹³C NMR (101 MHz, DMSO-d₆) δ 158.77, 156.68, 149.91, 134.92, 131.40, 129.49, 124.62, 123.41, 123.34, 123.13, 117.37, 111.15, 111.05; mass (m/z): 343 (M⁻+ 1), 342 (M⁻), and 100 (100%, base peak). Anal. calc. for C₃₆H₂₆Cl₂N₄ (342.58): C, 50.98 (49.24); H, 2.06 (2.18); N, 12.27 (12.44).

4.1.4. General procedure for synthesis of the target compounds 13a,b. A mixture of 2,4,5-trichloroquinazoline 10a or 2,4,6-trichloroquinazoline 10b (0.25 g, 1.07 mmol), Et₃N (0.34 mL, 2.36 mmol) and 3-aminopiperidine-2,6-dione hydrochloride 12 (0.19 g, 1.18 mmol) in isopropanol (20 mL) was stirred at r.t. for 8 h. The obtained precipitate was collected by filtration, washed with water, dried and crystallized from ethanol to give the corresponding 4-aminquinazoline derivatives (13a,b), respectively.

4.1.4.1. 3-[(2,5-Dichloroquinazolin-4-yl)amino]piperidine-2,6-dione 13a.

Solid (yield, 51.70%); m.p. = 218–220 ºC. IR (KBr, cm⁻¹): 3344, 3195 (2NH), 3088 (CH aromatic), 2855 (CH aliphatic), 1698 (C=O imide); ¹H NMR (DMSO-d₆) δ ppm: 11.04 (s, 1H, CONHCO), 8.72 (d, J = 8.1 Hz, 1H, CONH), 7.76 (m, 1H, Ar-H), 7.63 (m, 2H, Ar-H), 5.07 (m, 1H, CH-piperidine), 3.13 (m, 1H, CH₂CONH-piperidine), 2.81 (m, 1H, CH₂CONH-piperidine), 2.56 (m, 1H, CH₂CONH-piperidine); anal. calc. for C₁₃H₁₂Cl₂N₂O (315.15): C, 48.02 (48.28); H, 3.10 (3.34); N, 17.23 (17.41).

4.1.4.2. 3-[(2,6-Dichloroquinazolin-4-yl)amino]piperidine-2,6-dione 13b.

Solid (yield, 45.96%); m.p. = 198–200 ºC. IR (KBr, cm⁻¹): 3316, 3186 (2NH), 3093 (CH aromatic), 2875 (CH aliphatic), 1725 (C=O imide); 10.99 (s, 1H, CONHCO), 9.02 (d, J = 8.2 Hz, 1H, CONH), 8.47 (s, 1H, Ar-H), 7.89 (d, J = 8.9 Hz, 1H, Ar-H), 7.71 (d, J = 8.9 Hz, 1H, Ar-H), 5.19 (m, 1H, CH-piperidine), 2.90 (m, 1H, CH₂CONH-piperidine), 2.62 (m, 1H, CH₂CONH-piperidine), 2.25 (m, 1H, CH₂CONH-piperidine), 2.11 (m, 1H, CH₂CONH-piperidine); ¹³C NMR (101 MHz, DMSO-d₆) δ 173.38, 171.97, 160.98, 157.32, 149.60, 134.72, 130.91, 129.39, 123.00, 114.76, 51.18, 31.36, 29.35; mass (m/z): 327 (M⁻+ 2), 326 (M⁻+ 1), 325 (M⁻), and 304 (100%, base peak). Anal. calc. for C₁₃H₁₀Cl₂N₂O₂ (325.15): C, 48.02 (48.25); H, 3.10 (3.31); N, 17.23 (17.59).

4.1.5. General procedure for synthesis of the target compounds 15a,b. A mixture of 2,4,5-trichloroquinazoline or 2,4,6-trichloroquinazoline (0.25 g, 1.07 mmol), Et₃N (0.19 mL, 1.28 mmol) and semicarbazide (0.01 g, 1.28 mmol) in isopropanol (20 mL) was stirred at r.t. for 8 h. After the reaction was complete (monitored by TLC), the obtained precipitate was collected by filtration, washed with water, dried and crystallized from ethanol to give the corresponding 4-aminquinazoline derivatives (VIIIₐ,b), respectively.
4.1.5.1. 2-(2,5-Dichloroquinazolin-4-yl)hydrazine-1-carboxamide 15a.

Solid (yield, 75.51%); m.p. = 189–191 °C. IR (KBr, cm⁻¹): 3457, 3349, 3374, 3194 (br, 4NH), 3050 (CH aromatic); ¹H NMR (DMSO-d6) δ ppm: 9.87 (s, 1H, NHNHSO₂), 8.45 (s, 1H, NHNHSO₂), 7.70 (d, J = 8.2 Hz, 1H, Ar−H), 7.56 (d, J = 8.00 Hz, 1H, Ar−H), 7.45 (m, 1H, Ar−H), 6.25 (s, 2H, NH₂); anal. calcd for C₉H₇Cl₂N₅O (272.09): C, 39.73 (39.88); H, 2.59 (2.75); N, 25.74 (25.53).

4.1.5.2. 2-(2,6-Dichloroquinazolin-4-yl)hydrazine-1-carboxamide 15b.

Solid (yield, 68.65%); m.p. = 171–173 °C. IR (KBr, cm⁻¹): 3471, 3426, 3267, 3190 (br, 4NH), 3027 (CH aromatic); ¹H NMR (DMSO-d6) δ ppm: 10.43 (s, 1H, NHNHSO₂), 8.45 (s, 1H, NHNHSO₂), 8.24 (d, J = 8.2 Hz, 1H, Ar−H), 7.88 (d, J = 8.4 Hz, 1H, Ar−H), 12.72 (m, 1H, Ar−H), 6.20 (s, 2H, NH₂); ¹³C NMR (101 MHz, DMSO-d6) δ ppm: 163.81, 157.71, 151.74, 139.83, 129.83, 123.26, 113.78; mass (m/z): 274 (M⁺ + 2), 272 (M⁺), and 127 (100%, base peak); anal. calcd for C₉H₇Cl₂N₅O (272.09): C, 39.73 (39.90); H, 2.59 (2.78); N, 25.74 (25.91).

4.1.6. General procedure for synthesis of the target compounds 19a,b. To a mixture of 2,5-dichloro-4-hydrazonequinazoline (17) (0.25 g, 1.09 mmol) and Et₃N (0.19 mL, 1.31 mmol) in DMF, an appropriate benzenesulfonyl chloride (1.20 mmol) namely; 4-methylbenzenesulfonyl chloride 19a and 4-fluorobenzenesulfonyl chloride 19b was added dropwise in ice salt bath. The reaction mixture was stirred for 2 h. The reaction mixture was poured into crushed ice to give a precipitate. The formed precipitate was collected by filtration, washed with water, dried and crystallized from ethanol to afford the corresponding benzenesulfonylhydrazide derivatives (19a,b), respectively.

4.1.6.1. N′-(2,5-Dichloroquinazolin-4-yl)-4-methylbenzenesulfonylhydrazide 19a.

Solid (yield, 83.68%); m.p. = 155–157 °C. IR (KBr, cm⁻¹): 3397, 3306 (2NH), 3086 (CH aromatic), 1163 (SO₂ group); ¹H NMR (DMSO-d6) δ ppm: 10.04 (s, 1H, NHNHSO₂), 7.82 (s, 1H, NHNHSO₂), 7.78 (s, 1H, Ar−H), 7.70 (d, J = 8.4 Hz, 1H, Ar−H), 7.64 (d, J = 8.3 Hz, 1H, Ar−H), 7.53 (d, J = 8.2 Hz, 1H, Ar−H), 7.46 (m, 1H, Ar−H), 7.33 (d, J = 8.0 Hz, 2H, Ar−H), 2.31 (s, 3H, CH₃); anal. calcd for C₁₅H₁₃ClN₂O₄S (383.25): C, 57.01 (56.89); H, 3.48 (3.52); N, 16.20 (16.32).

Solid (yield, 80.35%); m.p. = 235–237 °C. IR (KBr, cm⁻¹): 3330, 3188 (2NH), 3062 (CH aromatic), 2921 (CH aliphatic), 1166 (SO₂ group); ¹H NMR (DMSO-d6) δ ppm: 11.72 (s, 1H, NHNHSO₂), 9.24 (s, 1H, CNHNSO₂), 7.98 (d, J = 7.8 Hz, 1H, Ar−H), 7.83 (s, 1H, Ar−H), 7.79 (m, 4H, Ar−H), 7.42 (d, J = 7.9 Hz, 2H, Ar−H), 2.40 (s, 3H, CH₃); anal. calcd for C₁₅H₁₃ClN₂O₄S (383.25): C, 51.65 (51.89); H, 3.76 (3.85); N, 16.06 (16.32).

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RSC Adv., 2022, 12, 33525–33539 | 33535
4.2. Biological testing

4.2.1. In vitro antitumor assay. This test was carried out on three different human cancer cell lines: MCF-7, HCT116, and HepG2 using the MTT method as described in ESI.†

4.2.2. Estimation of TNF-α, CASP8, and VEGF in HepG2 cells supernatant. The levels of TNF-α, CASP8, and VEGF in cell culture supernatants were estimated by ELISA technique as described in ESI.†

4.2.3. Estimation of nuclear factor kappa-B (NF-κB) in HepG2 cell lysate. Anti-rabbit NF-κB P65 polyclonal antibody was measured using the ELISA plate reader in cell lysate as described in ESI.†

4.2.4. Cytotoxicity against Vero cell line. This test was carried out using MTT method on Vero cell line as described in ESI.†

4.2.5. In silico studies

4.2.5.1. Pharmacokinetic profiling study. This study was applied using Discover studio 4.0 as described in ESI.†

4.2.5.2. ADMET studies. ADMET descriptors were determined using Discover studio 4.0 as described in ESI.†

4.2.5.3. Toxicity studies. The toxicity parameters of the synthesized compounds were calculated using Discovery studio 4.0 as described in ESI.†

Conlicts of interest

There is no conflict of interest.

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