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Synthesis of novel phthalazine-based derivatives with potent cytotoxicity against HCT-116 cells through apoptosis and VEGFR2 inhibition†

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The parent ethyl 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoate (**3**) has 25 compounds. Their respective mono, dipeptides and hydrazones derivatives were produced by chemoselective *N*-alkylation *via* addition reaction of 4-benzylphthalazin-1(2H)-one (**2**) with ethyl acrylate and anhydrous potassium carbonate to give ethyl 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoate (**3**). The ester **3** was hydrazinolized to give the corresponding hydrazide 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanehydrazide (**5**), then azide **6** coupled with amino acid ester hydrochloride and/or amines to afford several parent esters **8a–c**, then a series of hydrazinolized reactions occurred to give corresponding hydrazides **9a–c**. The hydrazide **9a** was subjected to the azide coupling procedure, which resulted in the formation of various dipeptides. Subsequently, it was condensed with various aldehydes to yield hydrazone derivatives **13a–d**. Interestingly, compounds **9c**, **12b**, and **13c** exhibited potent cytotoxicity with IC_{50} values of 1.58, 0.32 and 0.64 μ M compared to sorafenib (IC_{50} = 2.93 μ M). Compound **12b** exhibited potent VEGFR2 inhibition by 95.2% with an IC_{50} value of 17.8 μ M compared to sorafenib (94.7% and IC_{50} of 32.1 μ M). For apoptosis activity, **12b**-treatment induced apoptosis in HCT-116 cells by 21.7-fold, arresting the cell proliferation at S-phase. Finally, it formed a good binding affinity towards VEGFR2 protein with a binding energy of -10.66 kcal mol $^{-1}$, and it formed binding interactions with the key interactive amino acids.

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Introduction

Generally, cancer seems to be the leading cause of death in high- and upper-middle-income countries¹ and the second most common cause of death after cardiovascular disease.² According to previous studies, around 18.1 million new cases of cancer were detected, with lung cancer accounting for 18.4%, followed by breast (11.6%), prostate (7.1%), colorectal (6.1%),

stomach, and liver cancer.^{3,4} About 10 million people died of cancer in 2020, while 19.3 million new cases were identified.⁵ Cancer occurs when abnormal cells divide rapidly and spread to other parts of the body and tissues, finally forming a tumor.⁴ The toxicity and the side effects of present antineoplastic drugs, along with the appearance of drug resistance, are the Major drawbacks of chemotherapy.⁶ Despite advances in our understanding of the biochemical processes involved in carcinogenesis and fifty years of chemotherapy research, there are still many obstacles to overcome before cancer treatments can be considered effective. These include diversity in tumor cells, drug resistance, therapy-related side effects, and the limitations of animal models.⁷ Cancer chemotherapy has been developed for molecular treatments that are more selective and do not have the toxicity of typical cytotoxic drugs.⁸ Heterocyclic compounds have been applied to treat a variety of diseases, including cancer. Biological molecules in our body, such as DNA, RNA, and vitamins, contain heterocyclic core rings, which make heterocyclic compounds advance significantly in the medicinal field.^{4,9} Hydrazine-containing compounds have attracted much attention due to their pharmacological properties and clinical uses.^{10,11} Hydrazides are an essential class of chemicals for novel medication development because they

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contain H-bond donors/acceptors that can create H-bonds with their recipients within the target protein active sites.¹² Phthalazin-1(2H)-one derivatives are a class of diaza heterocycles known for their potential medical applications. Thus, this class of compounds has been shown to have a wide range of biological properties, such as anti-diabetic and anti-cancer.¹³ Over the last two decades, there has been a significant focus on producing many phthalazines as promising drug targets for cancer treatment¹⁴ and other biological activity, as shown in (Fig. 1). The phthalazine derivative azelastine **1** is an antihistamine used to treat allergic rhinitis.¹⁵ Zopolrestat **5** is a phthalazinone derivative that has been examined in clinical studies. It inhibits aldose reductase and has the potential to prevent retinopathy, neuropathy, and cataract formation in diabetes.¹⁶ The aminophthalazine and hydrazinylphthalazine moiety can also be found in the core of many commercial drugs, such as hydralazine **2**,¹⁷ carbazeran **6**,¹⁸ and budralazine, which are used for the treatment of heart failure, as well as in the structure of the effective anti-cancer drugs.¹⁹ Moreover, in recent years, there has been interest in using several VEGFR-2 inhibitors for targeted cancer therapy, which contain phthalazinone derivative such as vatalanib **3**, ZD 6474 **4**,²⁰ and other compounds 1-(4-chlorophenyl)-3-(4-((4-chlorophthalazin-1-yl)amino)phenyl)urea (**7**), and 1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(4-((4-chlorophthalazin-1-yl)oxy)phenyl)urea (**8**) which showed the significant inhibitory effects.²¹

Accordingly, we aimed to design and synthesize novel phthalazine-based amine and amino acid derivatives with

characterization and purity, and to investigate their cytotoxicity against HCT-116 cells along with investigating both molecular target; vascular endothelial growth factor receptor 2 (VEGFR) with apoptosis-induction as the cell death mechanism.

Results & discussion

Recent studies^{15,22} revealed how to control chemoselective alkylation of amides and thioamides separately. As a follow-up to these results, we chose to apply them to the structure modification of 4-benzylphthalazin-1(2H)-one (**2**), our model heterocyclic amide. The addition reaction of the model nucleophile **2** with ethyl acrylate gave ethyl 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoate (**3**). According to Pearson's hard soft acid base principle, reaction control points like basicity and nucleophilicity of both N and O atoms determine how the addition reaction behaves toward electrophiles. Instead of occurring on the O atom or even at both atoms in a competitive reaction, this reaction only happens on the N atom. The resulting chemoselective *N*-alkylation reaction can be effectively interpreted as the result of the interaction between the high-energy HOMO at the nitrogen atom of the nucleophile and the low-energy LUMO of the electrophile, which creates a narrow energy gap and high reactivity that ultimately results in *N*-alkylation.²³ The ester **3** interacted with either sodium hydroxide or hydrazine hydrate to form 3-((4-benzyl-1-oxophthalazin-2(1H)-yl) propanoic acid (**4**) and 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanehydrazide (**5**). The acetic acid

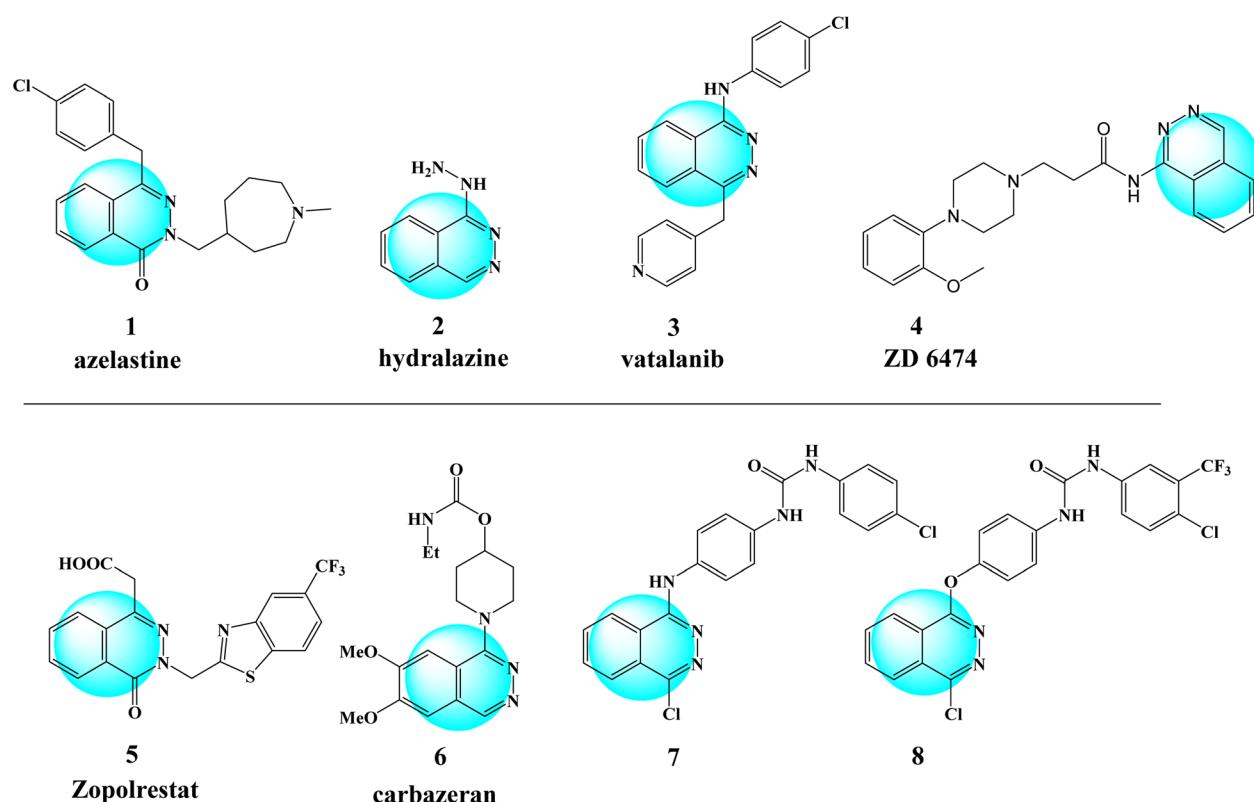


Fig. 1 Structure of biologically active phthalazine derivatives.

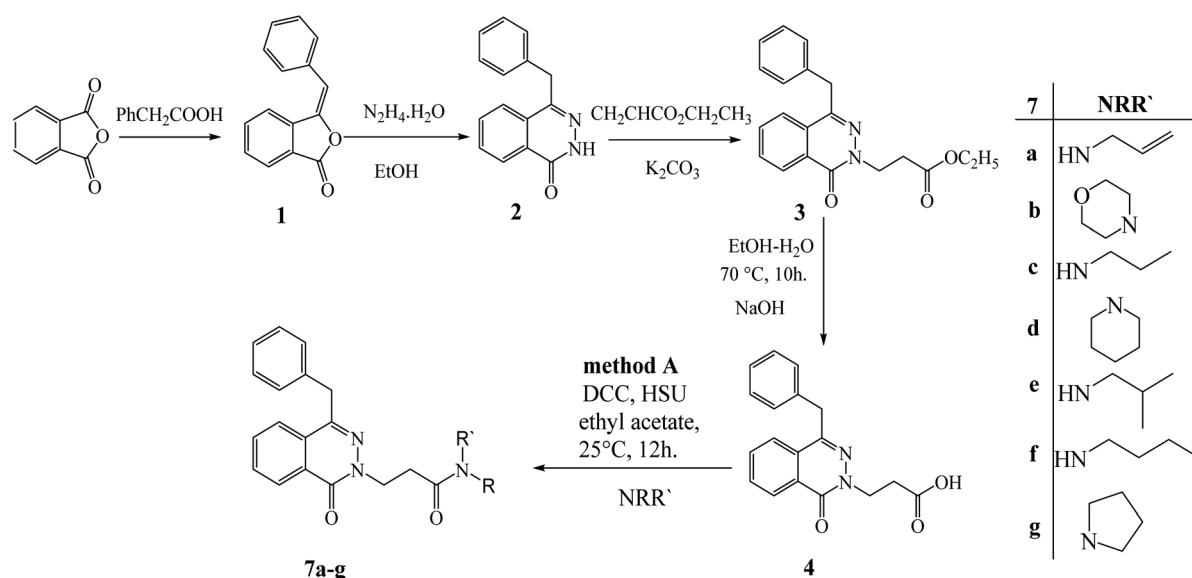


derivative **4** and hydrazide **5** are very interesting precursors for modifying the structure of 4-benzylphthalazin-1(*2H*)-one (**2**) by attaching amines or amino acids *via* peptide bond using either *N,N'*-dicyclohexylcarbodiimide (DCC) or azide coupling conditions. At ambient temperature, 3-(4-benzyl-1-oxophthalazin-2(*1H*)-yl) propanoic acid (**4**) reacted with various amines under DCC conditions, and produced 2-(4-benzyl-1-oxophthalazin-2(*1H*)-yl)-*N*-alkyl-propanamide **7a–g** (Scheme 1).

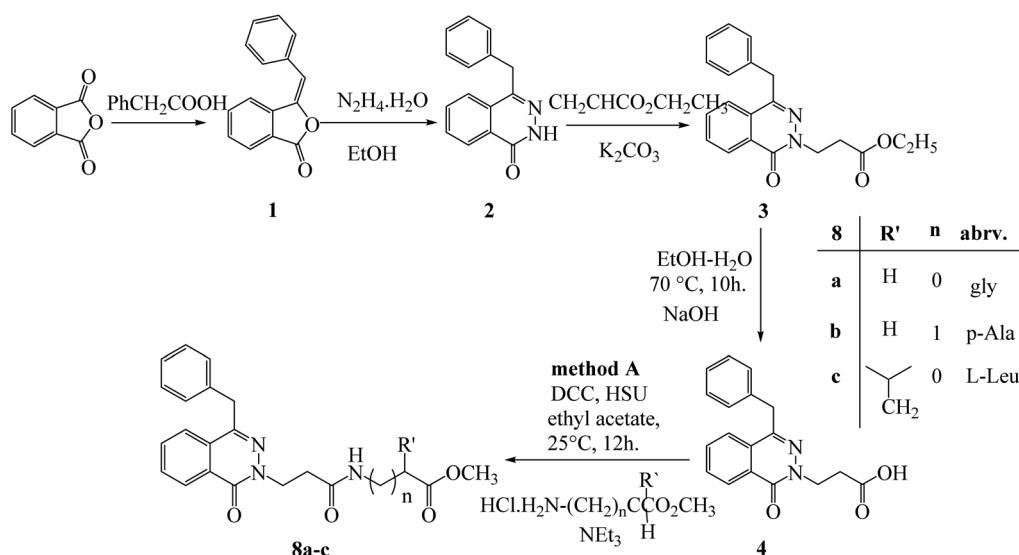
Methyl (3-[4-benzyl-1-oxophthalazin-2(*1H*)-yl] propanoyl amino) alkanoates **8a–c** were obtained *via* reaction of 3-(4-benzyl-1-oxophthalazin-2(*1H*)-yl) propanoic acid (**4**) with different amino acid ester hydrochloride under DCC conditions (Scheme 2).

3-(4-Benzyl-1-oxophthalazin-2(*1H*)-yl) propanehydrazide (**5**) was obtained *via* reaction of ester **3** with hydrazine hydrate in

ethanol under reflux for 6 h. Under azide coupling condition, hydrazide **5** was reacted with different amines in presence of NaNO_2/HCl ; the amide derivatives **7a–g** were obtained (Scheme 3). The ^1H NMR spectra of all compounds displayed a sharp singlet signal around 4.30 ppm for ($\text{CH}_2\text{-Ph}$), a multiplet peaks around 4.62–4.36 ppm for ($\text{CH}_2\text{CH}_2\text{CO}$), a multiplet peaks around 2.95–2.85 ppm for ($\text{CH}_2\text{CH}_2\text{CO}$) and the aromatic protons appeared between 8.46 and 7.15 ppm. ^{13}C NMR spectra revealed the methylene carbon of the ($\text{CH}_2\text{-Ph}$) group at 38 ppm. While ($\text{NCH}_2\text{CH}_2\text{CO}$) (attached to N-2) appeared between 47.0 and 46.0 ppm, ($\text{CH}_2\text{CH}_2\text{CO}$) appeared between 30.0 and 35.0 ppm, all the aromatic carbons were found between 145.85 and 125.00 ppm. The carbonyl group of the phthalazinone ring was observed around 158.68 ppm. The additional significant

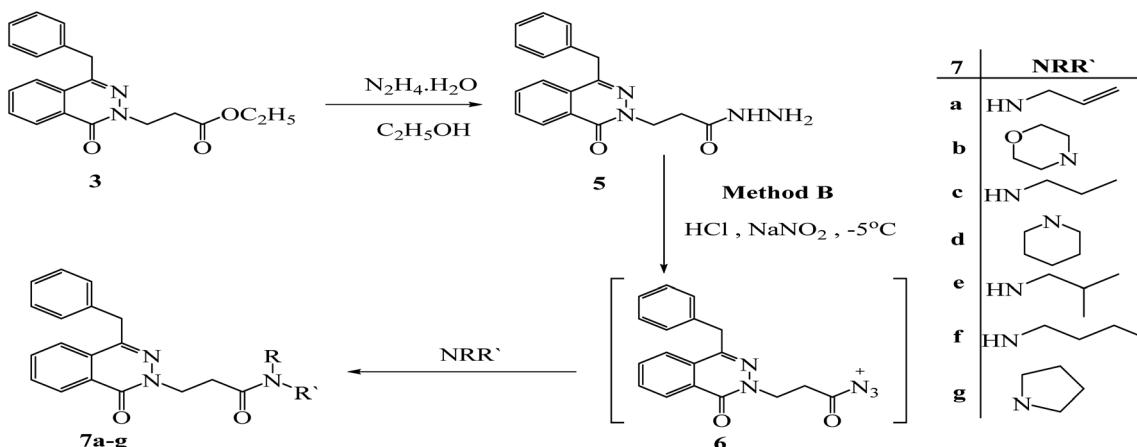


Scheme 1 Preparation of 2-(4-benzyl-1-oxophthalazin-2(*1H*)-yl)-*N*-alkyl-propanamide **7a–g** by method (A).



Scheme 2 Preparation of methyl (3-[4-benzyl-1-oxophthalazin-2(*1H*)-yl] propanoyl amino) alkanoates **8a–c** by method (A).





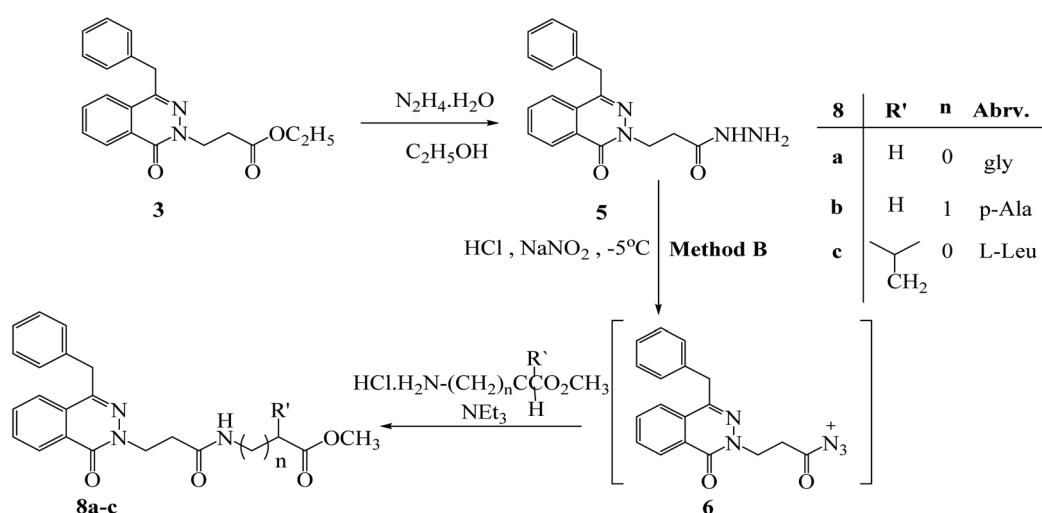
Scheme 3 Preparation of 2-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-alkyl-propanamide 7a-g by method (B).

data could be discussed as follows: the ^1H NMR spectrum of ester 3 gave additional signals at 4.05–3.99 ppm multiplet peaks for OCH_2CH_3 , and 1.09 ppm triplet peaks for CH_3 . The ^{13}C -NMR spectrum has signals at 171.35 for the carbonyl group of esters, peaks at 60.53, and 14.35 ppm for CH_2CH_3 & CH_3 respectively. The IR showed the presence of $2\text{C}=\text{O}$ bands at 1720 and 1639 cm^{-1} . The ^1H NMR of hydrazide 5 showed the hydrazino (NHNH_2) group protons at 4.18 ppm for NH_2 and 9.11 for NH . The IR showed the presence of NHNH_2 bands at 3300 and 3194 cm^{-1} . ^1H NMR and ^{13}C NMR were used to elucidate the synthetic construction of *N*-allyl-3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanamide (7a), which yielded the following signals: the olefinic methylene protons ($\text{CH}=\text{CH}_2$) appeared as two doublet at 5.06 ppm for the *cis* proton with coupling constant values $J_{\text{cis}} = 10.4$, while the *trans* proton appeared at 5.14 ppm with coupling constant $J_{\text{trans}} = 17.4$. The olefinic CH ($\text{CH}=\text{CH}_2$) appeared as multiplet at 5.85–5.75 ppm and NHCH_2 appeared as triplet at 4.59 ppm; the ^{13}C -NMR spectrum has signals at 134.17, 116.32, 42.01 ppm for (CHCH_2), (CHCH_2) and

(CH_2CHCH_2) respectively. The NMR spectrum of compound 7b showed multiplet signals at 3.67–3.65 ppm for 2OCH_2 and 3.53–3.50 ppm for 2NCH_2 in morpholine moiety. And the corresponding carbons appeared at 66.85, 66.65 ppm for ($2\text{CH}_2\text{O}$), and 46.03, 41.93 ppm for ($2\text{CH}_2\text{N}$).

Hydrazide 5 was reacted with different amino acid hydrochloride in presence of NaNO_2/HCl ; methyl (3-[4-benzyl-1-oxophthalazin-2(1H)-yl] propanoyl amino) alkanoates 8a–c were obtained (Scheme 4).

The azide method gave the same product as DCC-method but with higher yield 60–78%. So, hydrazide 5 is used as a starting point to create new phthalazinone compounds with significant biological activity. By attaching another amino acid *via* a peptide bond applying an azide condition. The azide technique is a well-known peptide synthesis technique that minimizes racemization while avoiding interferometer byproduct.²⁰ The esters 8a–c was believed to be a major stage in the chemical structure modification of the phthalazinone nucleus. Hydrazides 9a–c were obtained *via* reaction of esters



Scheme 4 Preparation of methyl (3-[4-benzyl-1-oxophthalazin-2(1H)-yl] propanoyl aminol alkanoates 8a-c by method (B).

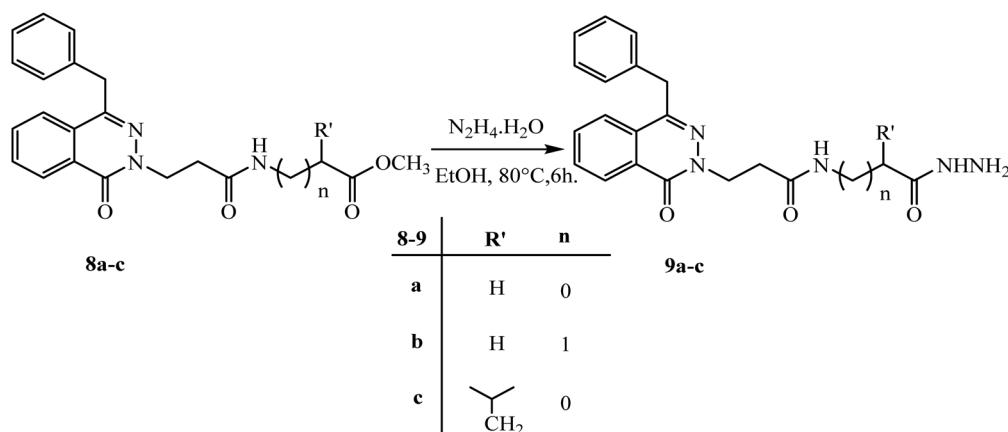


8a–c with hydrazine hydrate in ethanol under reflux for 6 h (Scheme 5). The compound **8a** has the ^1H -NMR spectrum of characteristic following signals: a multiplet signals at 4.04–4.01 ppm of NHCH_2CO and a singlet peak at 3.67 ppm of OCH_3 . The ^{13}C -NMR spectrum has signals at 170.69, 52.13, and 41.28 ppm for ($\text{C}=\text{O}$) ester, (OCH_3), and (NHCH_2), respectively. Compared with compound **9a**, the signal of OCH_3 disappeared, and new signals developed as broad signals at 9.07 and 3.40 ppm for (NH_2NH_2) and (NH_2), respectively. Also, The IR showed the presence of NH_2NH_2 bands at 3292 and 3200 cm^{-1} which confirm the formation of new hydrazide.

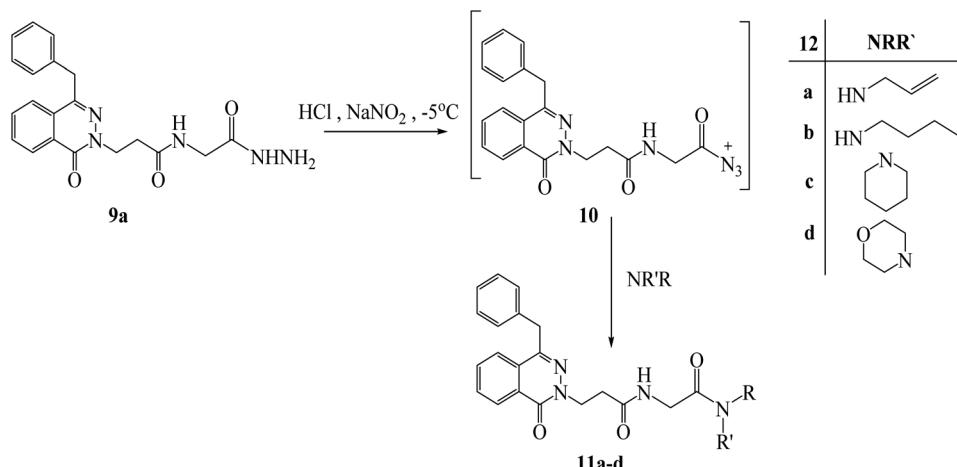
Under azide coupling conditions, 3-(4-benzyl-1-oxophthalazin-2(1*H*)-yl)-*N*-(2-hydrazinyl-2-oxoethyl)propanamide (**9a**) was reacted with different amines such as allyl, *n*-butyl, piperidin, and morpholine and obtained *N*-substituted-3-((4-benzyl-1-oxophthalazin-2(1*H*)-yl)-2-oxoethyl) propanamides **11a–d** (Scheme 6). In respect to compound **7a**, compound **11a** showed additional peaks for NHCH_2CO as multiplet at 3.97–3.95 in ^1H NMR, and signals at 169.00 and 43.59 ppm for ($\text{C}=\text{O}$) and (NHCH_2CO) in ^{13}C NMR. The IR showed the addition of new carbonyl at 1630 cm^{-1} . The structure of compound **11c** was

confirmed from ^1H NMR which showed additional signals as following a multiplet at 3.55–3.52 ppm for NCH_2 , a multiplet at 3.31–3.28 ppm for NCH_2 , a quartet signals at 1.63 ppm for $\text{CH}_2\text{CH}_2\text{CH}_2$, and a triplet signal at 1.53 ppm for $\text{CH}_2\text{CH}_2\text{CH}_2$) which confirmed the presence of piperidine ring, and the corresponding carbons showed signals at 45.42, 43.11, 24.31, 26.11 and 25.36 ppm respectively. Compound **11d** exhibited additional peaks for NHCH_2CO as multiplet at 4.03–4.02 ppm and corresponding carbon at 41.17 ppm compared to compound **7b**.

Similarly, hydrazide **9a** was reacted with various amino acid methyl esters such as glycine, β -alanine, and leucine *via* azide coupling condition and produced dipeptide compounds **12a–c** with an appropriate yield (Scheme 7). The formation of dipeptide was confirmed by using different analysis such as ^1H NMR and ^{13}C NMR. The compound **12c** showed two multiplet at 7.44–7.42 and 7.18–7.15 for 2 NH , a multiplet at 4.12–3.93 ppm for NHCH_2CO , a singlet peak at 3.66 ppm for OCH_3 , a doublet at 1.61 ppm for CH_2CH , a triplet at 1.24 ppm for CH_2CH , and a multiplet at 0.92–0.87 ppm for 2 CH_3 . The ^{13}C -NMR spectrum has signals at 173.20, 171.36, and 169.22 ppm for three carbonyl groups, also showed signals at 52.16, 50.92, 41.01, 35.13, 24.80,

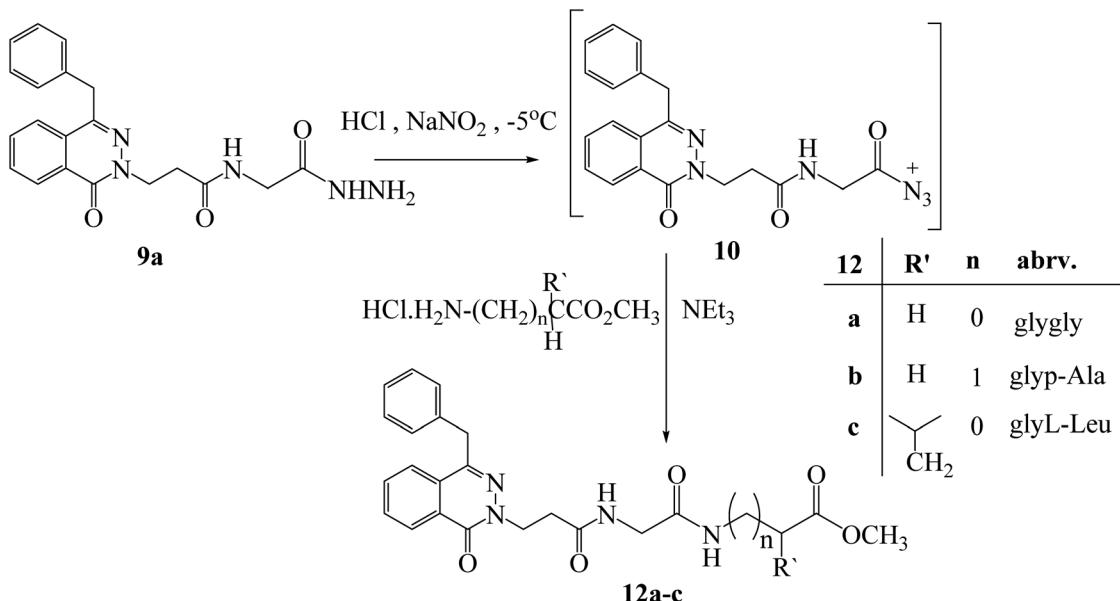


Scheme 5 Preparation of various 3-(4-benzyl-1-oxophthalazin-2(1*H*)-yl)-*N*-(2-hydrazinyl-2-oxoethyl)alkanamide **9a–c**.



Scheme 6 Preparation of *N*-substituted-3-((4-benzyl-1-oxophthalazin-2(1*H*)-yl)-2-oxoethyl) propanamides **11a–d**.





Scheme 7 Preparation of methyl-[3-(4-benzyl-1-oxo-1H-phthalazin-2-yl)-acetylamino] alkanoates 12a–c.

22.74, and 21.78 ppm for (NHCHCO), (OCH₃), (CH₂CH(CH₃)₂), (CH₂CH₂CO), (CH(CH₃)₂), and (2CH₃) respectively. The IR showed the presence of 2 (C=O) of dipeptide at 1645, and 1628 cm⁻¹.

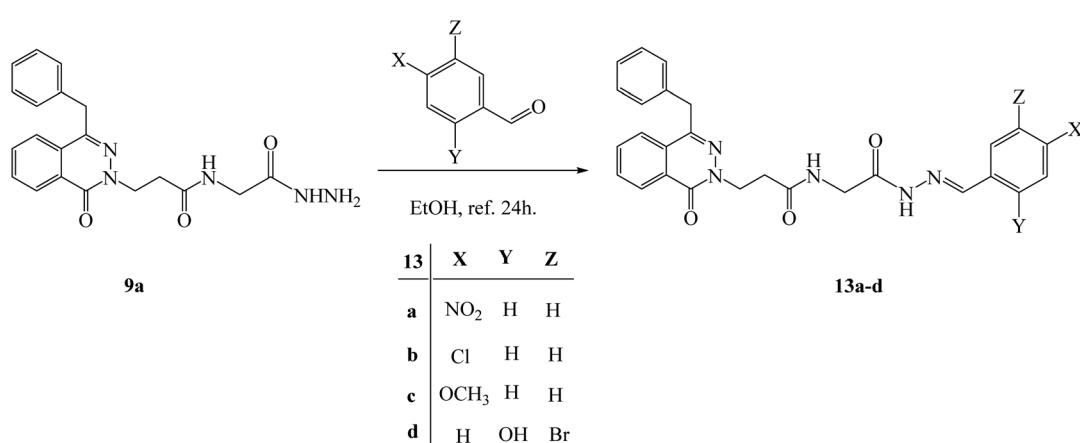
Condensation of the hydrazide 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-hydrazinyl-2-oxoethyl)propenamide (**9a**) with different aldehydes such as 4-nitro benzaldehyde, *p*-chloro benzaldehyde, anisaldehyde and 5-bromo-2-hydroxybenzaldehyde in ethanol under reflux 24 h; new hydrazone derivatives **13a–d** were obtained respectively in acceptable yields (Scheme 8). Finally, the formation of some hydrazones was confirmed by using different analysis such as ¹H NMR and IR. The compound **13a** show Z/E isomers mixture in 78/22 ratio. The NMR spectrum of compound **13a** showed a two singlet peaks at 11.71 and 11.68 ppm for CONHN, a two singlet peaks at 8.09 and 8.41 ppm for N=CH, and new aromatic at 7.96–7.81 ppm as a multiplet and 7.36 ppm as doublet. The IR

showed the presence of new H aromatic at 3208 and 3114 cm⁻¹, (NO₂) at 1524 cm⁻¹, and (C=N) at 1597 cm⁻¹.

Biological investigation

Cytotoxicity against HCT-116 cells

The MTT test was used to measure cytotoxic activity. This assay relies on metabolically active cells reducing a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT, to purple formazan crystals. The tested compounds were investigated for their cytotoxic activity against colon (HCT-116) cancer cells using the MTT assay (Fig. 2). As summarized in Table 1 with the IC₅₀ values. Interestingly, compounds **9c**, **12b**, and **13c** exhibited potent cytotoxicity with IC₅₀ values of 1.58, 0.32 and 0.64 μM compared to sorafenib (IC₅₀ = 3.23 μM). Compounds **5**, **11a**, and **7f** exhibited moderate cytotoxicity with IC₅₀ values range of 7.98–18.8 μM, while other

Scheme 8 Synthesis of some hydrazone derivatives **13a–d**.

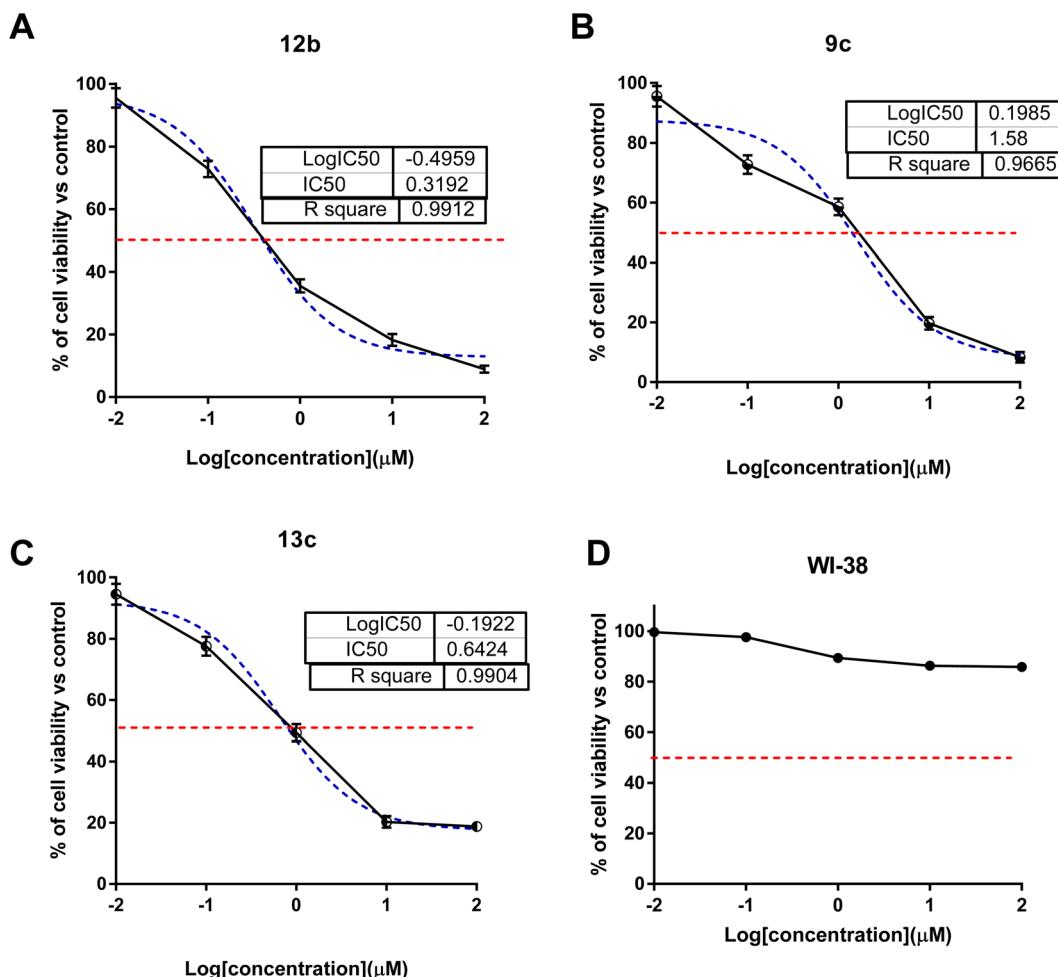


Fig. 2 Cell viability versus log concentrations of compounds 9c, 12b, and 13c (A–C) against cancer HCT-116 cells, while (D) is the cytotoxicity against 12b against WI-38 normal cells using MTT assay. Values are expressed as mean \pm SD of three independent values.

compounds showed poor cytotoxicity with higher IC_{50} values. Additionally, the most promising cytotoxic compounds weren't cytotoxic against normal WI-38 cells with higher IC_{50} values than 50 μ M. Hence, these compounds were worth testing for effective molecular target and apoptosis-induction activity.

Table 1 Cytotoxicity of the synthesized derivatives against HCT-116 cells using MTT assay

Compounds	IC_{50} (μ M) \pm SD ^a	Compounds	IC_{50} (μ M) \pm SD ^a
5	13.8 \pm 0.2	9c	1.58 \pm 0.21
7a	34.8 \pm 1.9	11a	7.98 \pm 0.7
7b	24.3 \pm 1.5	11d	\geq 50
7d	39.5 \pm 0.9	12b	0.32 \pm 0.01
7f	18.8 \pm 0.6	12c	48.7 \pm 1.6
8a	40.8 \pm 2.1	13a	45.7 \pm 1.8
8c	\geq 50	13c	0.64 \pm 0.05
9a	16.4 \pm 0.4	13d	
Sorafenib	3.23 \pm 0.03		

^a IC_{50} values were calculated as the average of three independent trials using a dose-response curve in GraphPad prism. NT = not tested.

VEGFR enzyme inhibition

VEGFR is one type of tyrosine kinase (TK) receptor, it was conducted through measuring the percentage of enzyme inhibition at different concentrations using Luminescent assay kit. Compounds 9c, 13c, and 12b were tested for VEGFR2 inhibition, as seen in Table 2. They had promising VEGFR2 inhibition percentages of 92.4, 95.2, and 96.4 with IC_{50} values of 21.8, 17.8, 19.8 nM compared to sorafenib with 94.7% and IC_{50} value of 32.1 nM. Hence, compound 12b exhibited potent VEGFR2 inhibition compared to sorafenib.

Apoptosis-induction activity

To investigate cells with apoptotic cell death, Annexin V/PI procedure was commonly utilized. Combination of propidium iodide (PI) with Annexin V, can distinguish between viable, apoptotic, and necrotic cells by measuring changes in plasma membrane permeability and integrity. Additionally, cell cycle analysis was conducted to measure the percentage of cells population at each stage. Compound 12b was investigated regarding the apoptosis-induction activity in HCT-116 cells (Fig. 3). It induced total apoptosis in HCT-116 cells by 27.57%

Table 2 Percentage of VEGFR2 inhibition with IC_{50} values for the most cytotoxic compounds

Compound	VEGFR2	
	% Of inhibition at [10 μ M]	IC_{50} [nM] \pm SD ^a
9c	92.4 \pm 1.9	21.8 \pm 1.8
12b	95.2 \pm 2.1	17.8 \pm 1.6
13c	96.4 \pm 2.8	19.8 \pm 0.6
Sorafenib	94.68 \pm 3.4	32.1 \pm 0.9

^a Values are expressed as an average of three independent replicates. IC_{50} values were calculated using sigmoidal non-linear regression curve fit of percentage inhibition against five concentrations of each compound.

compared to untreated cells (0.9%). It caused late apoptosis by 7.67% and early apoptosis by 19.9%. So, **12b**-treatment induced apoptosis by 21.7-fold. Regarding the cell phase at which cell proliferation was arrested, cell cycle analysis was performed; **12b** treatment caused cell cycle arrest at S-phase, increasing the cell population by 38.3% compared to 27.8% in the untreated cells. Cells in G2-phase were non-significantly increased from 15.6% to 20.4%. At the same time, cell population at G1-phase decreased from 56.5% to 41.27%.

Molecular docking studies

One of the structural bioinformatics tools that can be used to highlight the binding mode disposition of compounds towards

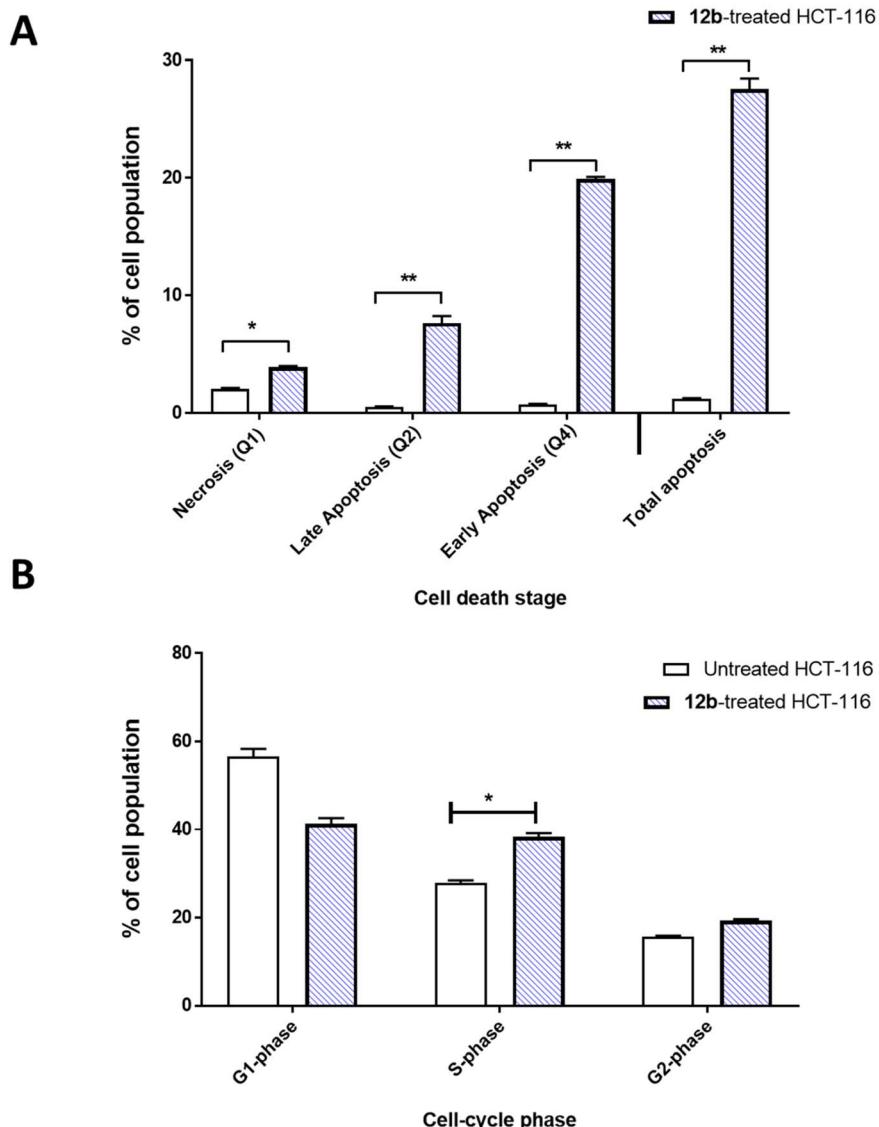


Fig. 3 Flow cytometry analysis for apoptosis/necrosis assessment in the untreated and **12b**-treated HCT-116 cells with the IC_{50} value of 0.32 μ M for 48 h. (A) Bar representation with cell percentage at each stage. (B) Bar representation for the cell cycle analysis reflecting the cell population in each phase "G1, S, and G" phases. Values are expressed as mean \pm SD of three independent trials $^*(P \leq 0.05)$, and $^{**}(P \leq 0.001)$ are significantly different using the un-paired test in GraphPad prism".



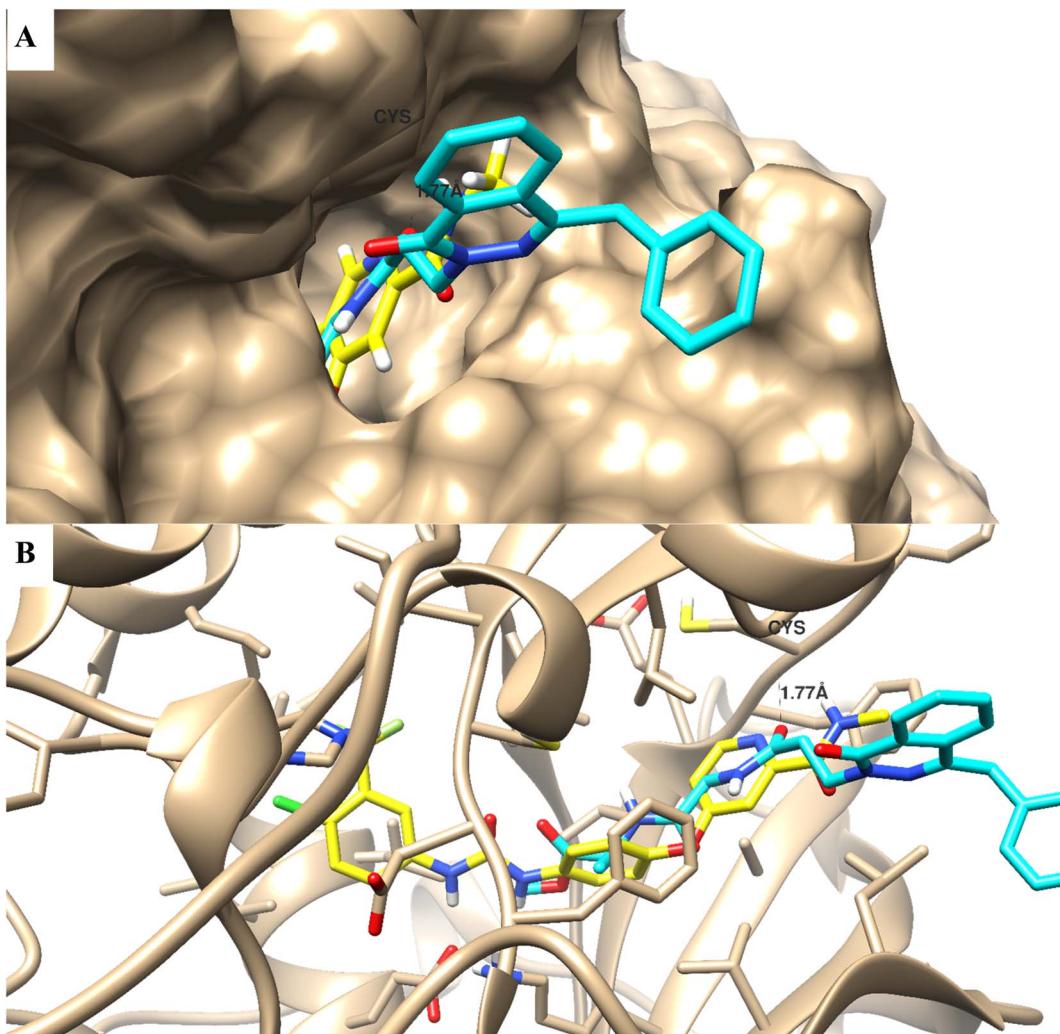


Fig. 4 Binding mode and ligand–receptor interactions of the co-crystallized ligand (yellow-colored) and compound **12b** (cyan-colored) inside the receptor binding site of VEGFR2 protein. (A) Surface presentation, and (B) interactive binding mode.

the protein active site, molecular docking study, was utilized. Compound **12b** was subjected to a molecular docking study to highlight the virtual mechanism of binding towards the VEGFR2 protein (Fig. 4); it maintained the biding mode disposition of the co-crystallized ligand; it was docked inside the VEGFR2 binding site with biding energy of $-10.66 \text{ kcal mol}^{-1}$, and it formed binding interactions with Cys 919 with bond length of 1.77 \AA , and it formed arene–arene interactions with Lys 838.

Experimental part

1-Chemistry

General procedures. The purity of the synthesized compounds was tested using thin layer chromatography (TLC) technique on silica gel 60 F₂₅₄ aluminum sheets (E. Merck, layer thickness 0.2 mm) in the following solvent systems; “ethyl acetate/petroleum ether (1 : 5) & ethyl acetate/petroleum ether (3 : 1)”, the spots on thin layer plates were detected by UV lamp. The melting points were determined using a Buchi 510 melting-

point system and are uncorrected. IR spectra were recorded in KBr on FTIR Mattson Spectrometers. Nuclear Magnetic Resonance (¹H-NMR & ¹³C-NMR) spectra were measured on Bruker spectrophotometer operating at (400 MHZ) using the appropriate deuterated solvents with chemical shift (δ) expressed in ppm downfield from TMS as internal standard at “nuclear magnetic resonance laboratory, Faculty of Science, Sohag University”. Elemental analyses were performed on a Flash EA-1112 instrument at the “Micro Analytical Laboratory, Faculty of Science, Cairo University, Egypt”. Compounds **1** and **2** were prepared according to the literature procedure.^{14,23}

Preparation of ethyl 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoate (3). A reaction of compound **2** (2.36 g, 0.01 mol), ethyl acrylate (2.0024 g, 0.02 mol) and anhydrous potassium carbonate (0.05 g, 0.01 mol) was refluxed for 48 h, cooled in ice and the white precipitate filtered.

White crystals; yield (1.62 g, 68%); mp: 88–90 °C; ¹H NMR (400 MHz, DMSO-*d*₆) (δ , ppm), (*J*, Hz): 8.28–8.25 (m, 1H, ArH), 7.89–7.86 (m, 1H, ArH), 7.82–7.75 (m, 2H, ArH), 7.33–7.30 (m, 2H, ArH), 7.27–7.23 (m, 2H, ArH), 7.18–7.15 (m, 1H, ArH), 4.42–

4.38 (m, 2H, CH_2CH_2CO), 4.27 (s, 2H, CH_2 -ph), 4.05–3.99 (m, 2H, CH_2CH_3), 2.84 (q, J = 6.4, 2H, CH_2CH_2CO), 1.09 (t, J = 6.8, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 171.35 (C=O) ester, 158.68 (C=O) ring, 145.53 (C-Ar), 138.45 (C-Ar), 133.73 (CH-Ar), 132.11 (CH-Ar), 128.95 (C-Ar & 2CH-Ar), 128.79 (2CH-Ar), 127.88 (C-Ar), 126.96 (CH-Ar), 126.82 (CH-Ar), 126.07 (CH-Ar), 60.53 (CH_2CH_3), 46.39 (CH_2CH_2CO), 38.22 (CH_2 ph), 33.19 (CH_2CH_2CO), 14.35 (CH_3). IR (KBr) (cm^{-1}) 3082 (H-Ar), 2978 (H-Al), 1720 (C=O) ring, 1639 (C=O) ester, 1579 (C=C). MS (MALDI, positive mode, matrix DHB) m/z : 359.41 (M + Na)⁺. Elemental Analysis calculated for $C_{20}H_{20}N_2O_3$ (336.4) C, 71.41; H, 5.99; N, 8.33 found: C, 71.45; H, 5.94; N, 8.37.

Procedure for preparation of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoic acid (4). The procedure used for hydrolysis of ester 3 was reported in previous work,²⁴ to a solution of ethyl 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoate (3) (3.3615 g, 1.0 mmol) in 70% ethyl alcohol (10 ml), NaOH (0.6 g, 1.5 mmol) and 10 ml H₂O were added, and the reaction mixture was heated under reflux for 10 h. The reaction mixture was cooled and acidification by dil. HCl. The precipitated residue was crystallized from ethyl alcohol.

White crystals; yield (74%); mp: 154–156 °C; 1H NMR (400 MHz, DMSO-*d*₆) (δ , ppm), (*J*, Hz): 8.28–8.25 (m, 1H, ArH), 7.89–7.86 (m, 1H, ArH), 7.82–7.75 (m, 2H, ArH), 7.33–7.30 (m, 2H, ArH), 7.27–7.23 (m, 2H, ArH), 7.18–7.15 (m, 1H, ArH), 4.42–4.38 (m, 2H, CH_2CH_2CO), 4.27 (s, 2H, CH_2 -ph), 2.84 (q, J = 6.4, 2H, CH_2CH_2CO).

Procedure for preparation of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanehydrazide (5). A mixture of 3 (3.3615 g, 0.01 mol) and hydrazine hydrate (0.5 ml, 0.01 mol) in ethanol (30 ml) was refluxed for 6 h. The separated solid was filtered off and recrystallized from ethanol to give compound 5.

Off-white crystals; yield (2.15 g, 63.97%); mp: 170 °C; 1H NMR (400 MHz, DMSO-*d*₆) (δ , ppm), (*J*, Hz): 9.11 (bs, 1H, D₂O exchangeable, NH), 8.29–8.26 (m, 1H, ArH), 7.92–7.90 (m, 1H, ArH), 7.84–7.79 (m, 2H, ArH), 7.36–7.34 (m, 2H, ArH), 7.29–7.26 (m, 2H, ArH), 7.21–7.18 (m, 1H, ArH), 4.40–4.35 (m, 2H, CH_2CH_2CO), 4.30 (s, 2H, CH_2 -ph), 4.18 (bs, 2H, D₂O exchangeable, NH₂), 2.62–2.58 (m, 2H, CH_2CH_2CO). ^{13}C NMR (101 MHz, DMSO) δ 169.75 (C=O), 158.59 (C=O) ring, 145.53 (C-Ar), 138.56 (C-Ar), 133.72 (C-Ar), 132.11 (CH-Ar), 129.04 (C-Ar & 2CH-Ar), 128.85 (2CH-Ar), 127.99 (C-Ar), 126.96 (CH-Ar), 126.84 (CH-Ar), 126.13 (CH-Ar), 47.33 (CH_2CH_2), 38.22 (CH_2 ph), 32.91 (CH_2CH_2). IR (KBr) (cm^{-1}): 3022, 2965, 3300 (N-H), 3194 (NH₂), 1739, 1643 (C=O), 1583. MS (MALDI, positive mode, matrix DHB) m/z : 345.39 (M + Na)⁺. Elemental Analysis calculated for $C_{18}H_{18}N_4O_2$ (322.4) C, 67.07; H, 5.63; N, 17.38 found: C, 67.02; H, 5.67; N, 17.35.

General procedure for preparation of 2-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-alkyl-propanamide 7a–g

Method A. DCC coupling. The procedure used for DCC-HSU (dicyclohexyl carbodiimide-hydroxysuccinimide) coupling was reported in previous work²⁴ using 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoic acid (4) (3.08 g, 10.0 mmol) and the same molar equivalents of DCC, HSU and amines. The pure product of 2-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-alkyl-propanamide

7a–g were obtained by column separation using petroleum ether/ethyl acetate 3 : 1 as eluent.

Method B. Azide coupling. A cold solution of propanehydrazide 5 (3.22 g, 10 mmol) at (−5 °C) in acetic acid (60 ml) and hydrochloric acid (5N, 30 ml) was added portion wise under stirring to a cold solution (0 °C) of sodium nitrite (0.7 g, 0.01 mol) in water (30 ml). After 30 minutes of stirring at the same temperature, the azide that was produced *in situ* was extracted using cold ethyl acetate. It was then washed several times with cold water and 5% Na₂CO₃. The azide 6 was utilized in the following stage without additional purification after drying over anhydrous sodium sulphate. After making the cold-dried azide solution beforehand, 12 mmol of various amines were added to it. Afterwards, the mixture was kept 24 h in the refrigerator and then at room temperature for another 24 h. The reaction mixture was filtered, and the filtrated solution washed with “0.1N HCl, 5% Na₂CO₃” and water then dried over anhydrous sodium sulphate, the solvent was evaporated in vacuum to give amides 7a–g.

Synthesis of N-allyl-3-(4-benzyl-1-oxophthalazin-2(1H)-yl)propanamide (7a). White crystals; yield (Method A 35%, Method B 60%); mp: 171 °C; 1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.43–8.41 (m, 1H, ArH), 7.75–7.68 (m, 3H, ArH), 7.32–7.30 (m, 4H, ArH), 7.24–7.20 (m, 1H, ArH), 6.56 (brs, 1H, D₂O exchangeable, NH), 5.85–5.75 (m, 1H, $CHCH_2$), 5.14 (d, *J*_{trans} = 17.2, 1H, $CHCH_2$), 5.06 (d, *J*_{cis} = 10.4, 1H, $CHCH_2$), 4.59 (t, *J* = 7.2, 2H, NHCH₂), 4.30 (s, 2H, CH_2 -ph), 3.89–3.86 (t, *J* = 6.4, 2H, CH_2CH_2CO), 2.89–2.86 (t, *J* = 7.8, 2H, CH_2CH_2CO). ^{13}C NMR (101 MHz, CDCl₃) δ 170.11 (C=O), 159.48 (C=O) ring, 145.72 (C-Ar), 137.68 (C-Ar), 134.17 ($CHCH_2$), 132.99 (CH-Ar), 131.31 (CH-Ar), 129.20 (C-Ar), 128.73 (2CH-Ar), 128.40 (2CH-Ar), 128.15 (C-Ar), 127.20 (CH-Ar), 126.78 (CH-Ar), 125.22 (CH-Ar), 116.32 (CHCH₂), 47.31 (CH_2CH_2CO), 42.01 (CH_2CHCH_2), 38.90 (CH_2 ph), 35.61 (CH_2CH_2CO). IR (KBr) (cm^{-1}): 3063, 2916, 3300, 2851 (H-Al), 3237 (H-ole), 1724, 1651, 1579. MS (MALDI, positive mode, matrix DHB) m/z : 370.43 (M + Na)⁺. Elemental analysis calculated for $C_{21}H_{21}N_3O_2$ (347.4) C, 72.60; H, 6.09; N, 12.10 found: C, 72.62; H, 6.05; N, 12.15.

Synthesis of 4-benzyl-2-(3-morpholino-3-oxopropyl) phthalazin-1(2H)-one (7b). Off-white crystals; yield (Method A 40%, Method B 62%); mp: 90 °C; 1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.45–8.43 (m, 1H, ArH), 7.75–7.69 (m, 3H, ArH), 7.31 (d, *J* = 8, 4H, ArH), 7.23–7.22 (m, 1H, ArH), 4.61–4.57 (m, 2H, CH_2CH_2CO), 4.30 (s, 2H, CH_2 -ph), 3.67–3.65 (m, 6H, 2CH₂O & CH₂N), 3.53–3.50 (m, 2H, CH₂N), 2.96–2.92 (m, 2H, CH_2CH_2CO). ^{13}C NMR (101 MHz, CDCl₃) δ 169.14 (C=O), 159.33 (C=O), 145.46 (C-Ar), 137.79 (C-Ar), 132.90 (CH-Ar), 131.24 (CH-Ar), 129.29 (C-Ar), 128.70 (2CH-Ar), 128.42 (2CH-Ar), 128.21 (C-Ar), 127.10 (CH-Ar), 126.74 (CH-Ar), 125.18 (CH-Ar), 66.85 (CH₂O), 66.65 (CH₂O), 48.00 (CH_2CH_2CO), 46.03 (CH₂N), 41.93 (CH₂N), 38.91 (CH₂ph), 31.73 (CH_2CH_2CO). IR (KBr) (cm^{-1}): 3025, 2965, (2924 & 2851) H-Al, 1661, 1630, 1579. MS (MALDI, positive mode, matrix DHB) m/z : 400.46 (M + Na)⁺. Elemental analysis calculated for $C_{22}H_{23}N_3O_3$ (377.4) C, 70.01; H, 6.14; N, 11.13 found: C, 70.06; H, 6.18; N, 11.18.



Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-propylpropanamide (7c). White crystals; yield (Method A 50%, Method B 69%); mp: 184 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.43–8.40 (m, 1H, ArH), 7.75–7.67 (m, 3H, ArH), 7.30 (d, *J* = 7.6, 4H, ArH), 7.23–7.21 (m, 1H, ArH), 6.57 (brs, 1H, D₂O exchangeable, NH), 4.57 (t, *J* = 7.2, 2H, CH₂CH₂CO), 4.30 (s, 2H, CH₂ph), 3.22–3.16 (m, 2H, NHCH₂), 2.85 (t, *J* = 7.2, 2H, CH₂CH₂CO), 1.53–1.43 (m, 2H, CH₂CH₃), 0.87 (t, *J* = 7.7, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 170.19 (C=O), 159.52 (C=O) ring, 145.77 (C-Ar), 137.68 (C-Ar), 137.55 (C-Ar), 133.00 (CH-Ar), 131.30 (CH-Ar), 129.19 (C-Ar), 128.71 (2CH-Ar), 128.39 (2CH-Ar), 128.12 (CH-Ar), 127.15 (CH-Ar), 126.77 (CH-Ar), 125.22 (CH-Ar), 47.36 (CH₂CH₂CO), 41.34 (CH₂CH₂CH₃), 38.90 (CH₂ph), 35.73 (CH₂CH₂CO), 22.69 (CH₂CH₂CH₃), 11.31 (CH₃). IR (KBr) cm⁻¹: 3296 (N-H), 3088, 2949, 2880 (H-Al), 1726, 1637, 1583. MS (MALDI, positive mode, matrix DHB) *m/z*: 372.45 (M + Na)⁺. Elemental analysis calculated for C₂₁H₂₃N₃O₂ (349.4) C, 72.18; H, 6.63; N, 12.03 found: C, 72.12; H, 6.68; N, 12.08.

Synthesis of 4-benzyl-2-(3-oxo-3-(piperidin-1-yl) propyl) phthalazin-1(2H)-one (7d). Off-white crystals; yield (Method A 55%, Method B 70%); mp: 80 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.44–8.42 (m, 1H, ArH), 7.73–7.67 (m, 3H, ArH), 7.28–7.27 (m, 4H, ArH), 7.21–7.19 (m, 1H), 4.60–4.56 (m, 2H, CH₂CH₂CO), 4.29 (s, 2H, CH₂ph), 3.58–3.55 (m, 2H, NCH₂), 3.44–3.42 (m, 2H, NCH₂), 2.95–2.85 (m, 2H, CH₂CH₂CO), 1.63–1.54 (m, 2H, 3CH₂). ¹³C NMR (101 MHz, CDCl₃) δ 168.68 (C=O), 159.30 (C=O) ring, 145.32 (C-Ar), 137.87 (C-Ar), 132.79 (CH-Ar), 131.12 (CH-Ar), 129.28 (C-Ar), 128.67 (2CH-Ar), 128.41 (2CH-Ar), 128.26 (C-Ar), 127.09 (CH-Ar), 126.69 (CH-Ar), 125.13 (CH-Ar), 48.14 (CH₂CH₂CO), 46.63 (NCH₂), 42.63 (NCH₂), 38.92 (CH₂ph), 31.97 (CH₂CH₂CO), 26.48 (CH₂CH₂CH₂), 25.53 (CH₂CH₂CH₂), 24.52 (CH₂CH₂CH₂). IR (KBr) cm⁻¹: 3057, 2927, 2851 (H-Al), 1734, 1657, 1579. MS (MALDI, positive mode, matrix DHB) *m/z*: 398.48 (M + Na)⁺. Elemental analysis calculated for C₂₃H₂₅N₃O₂ (375.5) C, 73.57; H, 6.71; N, 11.19 found: C, 73.52; H, 6.75; N, 11.14.

Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-isobutylpropanamide (7e). White crystals; yield (Method A 45%, Method B 65%); mp: 164 °C ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.46–8.43 (m, 1H, ArH), 7.78–7.70 (m, 3H, ArH), 7.31 (d, *J* = 7.2, 4H, ArH), 7.24–7.23 (m, 1H, ArH), 6.48 (brs, 1H, D₂O exchangeable, NH), 4.60–4.41 (m, 2H, CH₂CH₂CO), 4.30 (s, 2H, CH₂ph), 3.09–3.06 (m, 2H, CH₂CH), 2.96–2.86 (m, 2H, CH₂CH₂CO), 1.79–1.71 (m, 1H, CH₂CH), 1.27–0.86 (m, 6H, 2CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 170.18 (C=O), 159.56 (C=O) ring, 145.81 (C-Ar), 137.67 (C-Ar), 133.02 (CH-Ar), 131.32 (CH-Ar), 129.21 (C-Ar), 128.73 (2CH-Ar), 128.53 (CH-Ar), 128.39 (CH-Ar), 128.14 (C-Ar), 127.20 (CH-Ar), 126.79 (CH-Ar), 125.23 (CH-Ar), 47.38 (CH₂CH₂CO), 47.01 (CH₂CH), 38.93 (CH₂ph), 35.80 (CH₂CH₂CO), 29.66 (CH₃), 28.36 (CH₃), 20.06 (CH(CH₃)₂). IR (KBr) cm⁻¹: 3296 (N-H), 3090, 2957, 2865 (H-Al), 1730, 1639, 1583. MS (MALDI, positive mode, matrix DHB) *m/z*: 386.47 (M + Na)⁺. Elemental analysis calculated for C₂₂H₂₅N₃O₂ (363.5) C, 72.70; H, 6.93; N, 11.56 found: C, 72.73; H, 6.95; N, 11.59.

Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-butylpropanamide (7f). White crystals; yield (Method A 55%, Method B 67%); mp: 140 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.46–8.43 (m, 1H, ArH), 7.76–7.71 (m, 3H, ArH), 7.31 (d, *J* = 7.2, 4H, ArH), 7.24–7.21 (m, 3H, ArH), 6.47 (brs, 1H, D₂O exchangeable, NH), 4.59–4.55 (m, 2H, CH₂CH₂CO), 4.31 (s, 2H, CH₂ph), 3.28–3.20 (m, 2H, NHCH₂), 2.90–2.83 (m, 2H, CH₂CH₂CO), 1.30–1.26 (m, 2H, CH₂CH₂CH₃), 0.95–0.91 (m, 2H, CH₂CH₃), 0.85 (t, *J* = 7, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 170.13 (C=O), 159.58 (C=O) ring, 145.82 (C-Ar), 137.66 (C-Ar), 133.02 (CH-Ar), 131.33 (CH-Ar), 129.20 (C-Ar), 128.73 (2CH-Ar), 128.39 (2CH-Ar), 128.13 (C-Ar), 127.20 (CH-Ar), 126.78 (CH-Ar), 125.23 (CH-Ar), 47.31 (CH₂CH₂CO), 39.36 (NHCH₂), 38.92 (CH₂ph), 35.77 (CH₂CH₂CO), 29.66 (CH₂CH₂CH₃), 20.03 (CH₂CH₃), 13.64 (CH₃). IR (KBr) cm⁻¹: 3300, 3080, 2937, 2863 (H-Al), 1732, 1639, 1567. MS (MALDI, positive mode, matrix DHB) *m/z*: 386.47 (M + Na)⁺. Elemental analysis calculated for C₂₂H₂₅N₃O₂ (363.5) C, 72.70; H, 6.93; N, 11.56 found: C, 72.74; H, 6.96; N, 11.51.

Synthesis of 4-benzyl-2-(3-oxo-3-(pyrrolidin-1-yl) propyl) phthalazin-1(2H)-one (7g). Off-white crystals; yield (Method A 50%, Method B 64%); mp: 86 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.43–8.41 (m, 1H, ArH), 7.72–7.65 (m, 3H, ArH), 7.27–7.26 (m, 4H, ArH), 7.20–7.18 (m, 1H, ArH), 4.61–4.53 (m, 2H, CH₂CH₂CO), 4.27 (s, 2H, CH₂ph), 3.48–3.41 (m, 4H, CH₂NCH₂), 2.88–2.85 (m, 2H, CH₂CH₂CO), 1.94–1.79 (m, 4H, CH₂CH₂). ¹³C NMR (101 MHz, CDCl₃) δ 169.02 (C=O), 159.25 (C=O), 145.23 (C-Ar), 137.88 (C-Ar), 132.78 (CH-Ar), 131.10 (CH-Ar), 129.24 (C-Ar), 128.66 (2CH-Ar), 128.40 (2CH-Ar), 128.25 (C-Ar), 127.10 (CH-Ar), 126.67 (CH-Ar), 125.11 (CH-Ar), 47.54 (CH₂CH₂CO), 46.62 (NCH₂CH₂), 45.60 (NCH₂CH₂), 38.90 (CH₂ph), 33.31 (CH₂CH₂CO), 26.04 (NCH₂CH₂), 24.37 (NCH₂CH₂). IR (KBr) cm⁻¹: 3025, 2965, 2873 (H-Al), 1661, 1643, 1579. MS (MALDI, positive mode, matrix DHB) *m/z*: 384.46 (M + Na)⁺. Elemental analysis calculated for C₂₂H₂₃N₃O₂ (361.4) C, 73.11; H, 6.41; N, 11.63 found: C, 73.16; H, 6.44; N, 11.68.

General procedure for preparation of synthesis of methyl [3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoyl amino] alkanoates 8a–c

Method A. DCC coupling. The procedure used for DCC-HSU (dicyclohexyl carbodiimide-hydroxysuccinimide) coupling was reported in previous work²⁴ using 3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoic acid (4) (3.08 g, 10.0 mmol) and the same molar equivalents of DCC, HSU and amino acids methyl ester hydrochloride. The pure product of methyl [3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoyl amino]alkanoates 8a–c were obtained by column separation using petroleum ether/ethyl acetate 3 : 1 as eluent.

Method B. Azide coupling. An ice-cold solution of sodium nitrite (0.7 g, 0.01 mol) in water (30 ml) was gradually added to a cold solution of propane hydrazide (5, 3.22 g, 10 mmol) in acetic acid (60 ml) and hydrochloric acid (5N, 30 ml) while stirring. The temperature of the mixture was kept at –5 °C. After 30 minutes of stirring at the same temperature, the azide that was produced *in situ* was extracted using cold ethyl acetate. It was then washed several times with cold water and 5% Na₂CO₃.



After drying over anhydrous sodium sulphate, the azide (**6**) was used without further in the next step. Amino acids methyl ester hydrochloride (15 mmol); “glycine, β -Alanine, and L-Leucine” which were placed with triethyl amine (1 g, 10 mmol) in ethyl acetate solution at (-5 °C) for 15 minutes. Then the amino acid methyl ester hydrochloride solution was added to the previously prepared cold dried solution of the azide. Afterwards, the mixture was kept 24 h in the refrigerator and then at room temperature for another 24 h. After filtering the reaction mixture, the resulting solution was rinsed with 0.1N HCl, 5% Na_2CO_3 , and water. It was then dried over anhydrous sodium sulphate. The solvent was subsequently evaporated under vacuum, and the remaining ethyl acetate-petroleum ether substance was crystallized to produce esters **8a–c**.

*Synthesis of methyl (3-(4-benzyl-1-oxophthalazin-2(1H)-yl)propanoyl) glycinate (**8a**).* White crystals; yield (Method A 50%, Method B 70%); mp: 152 °C; ^1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.44–8.41 (m, 1H, ArH), 7.74–7.66 (m, 3H, ArH), 7.29–7.26 (m, 4H, ArH), 7.22–7.20 (m, 1H, ArH), 6.92 (brs, 1H, D_2O exchangeable, NH), 4.62–4.57 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}$), 4.30 (s, 2H, CH_2ph), 4.04–4.01 (m, 2H, NHCH_2CO), 3.67 (s, 3H, OCH_3), 2.94–2.89 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}$). ^{13}C NMR (101 MHz, CDCl_3) δ 170.69 (C=O), 170.23 (C=O), 159.43 (C=O) ring, 145.69 (C-Ar), 137.74 (C-Ar), 132.95 (CH-Ar), 131.27 (CH-Ar), 129.21 (C-Ar), 128.70 (2CH-Ar), 128.40 (2CH-Ar), 128.17 (C-Ar), 127.19 (CH-Ar), 126.74 (CH-Ar), 125.20 (CH-Ar), 52.13 (OCH_3), 47.13 ($\text{CH}_2\text{CH}_2\text{CO}$), 41.28 (NHCH_2), 38.84 (CH_2ph), 35.15 ($\text{CH}_2\text{CH}_2\text{CO}$). IR (KBr) cm^{-1} : 3308 (N-H), 3067, 2957, 2845 (H-Al), 1749, 1643, 1630 (C=O) ester, 1581. MS (MALDI, positive mode, matrix DHB) m/z : 402.43 (M + Na)⁺. Elemental analysis calculated for $\text{C}_{21}\text{H}_{21}\text{N}_3\text{O}_4$ (379.4) C, 66.48; H, 5.58; N, 11.08 found: C, 66.44; H, 5.53; N, 11.13.

*Synthesis of methyl 3-(3-(4-benzyl-1-oxophthalazin-2(1H)-yl)propanamido) propanoate (**8b**).* White crystals; yield (Method A 55%, Method B 69%); mp: 150 °C; ^1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.36–8.34 (m, 1H, ArH), 7.69 (d, *J* = 7.6, 1H, ArH), 7.64–7.60 (m, 2H, ArH), 7.25 (t, *J* = 8.4, 4H, ArH), 7.18–7.16 (m, 1H, ArH), 6.92–6.88 (m, 1H, D_2O exchangeable, NH), 4.55–4.51 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}$), 4.26 (s, 2H, CH_2ph), 3.56 (s, 3H, CH_3), 3.51–3.46 (m, 2H, NHCH_2CH_2), 2.82–2.78 (m, 2H, NHCH_2CH_2), 2.51–2.47 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}$). ^{13}C NMR (101 MHz, CDCl_3) δ 172.71 (C=O), 170.51 (C=O), 159.33 (C=O) ring, 145.64 (C-Ar), 137.73 (C-Ar), 132.95 (CH-Ar), 131.25 (CH-Ar), 129.10 (C-Ar), 128.70 (2CH-Ar), 128.37 (2CH-Ar), 128.04 (C-Ar), 127.05 (CH-Ar), 126.74 (CH-Ar), 125.23 (CH-Ar), 51.65 (OCH_3), 47.37 ($\text{CH}_2\text{CH}_2\text{CO}$), 38.86 (CH_2ph), 35.34 ($\text{NHCH}_2\text{CH}_2\text{CO}$), 34.97 ($\text{NHCH}_2\text{CH}_2\text{CO}$), 33.81 ($\text{CH}_2\text{CH}_2\text{CO}$). IR (KBr) cm^{-1} : 3408 (NH), 3065, 2965, (2916 & 2857) H-Al, 1720, 1663, 1655 (C=O) ester, 1585. MS (MALDI, positive mode, matrix DHB) m/z : 416.46 (M + Na)⁺. Elemental analysis calculated for $\text{C}_{22}\text{H}_{23}\text{N}_3\text{O}_4$ (393.4) C, 67.16; H, 5.89; N, 10.68 found: C, 67.14; H, 5.84; N, 10.64.

*Synthesis of methyl (3-(4-benzyl-1-oxophthalazin-2(1H)-yl)propanoyl) leucinate (**8c**).* White crystals; yield (Method A 60%, Method B 78%); mp: 100 °C; ^1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.45–8.42 (m, 1H, ArH), 7.74–7.68 (m, 3H, ArH),

7.29 (d, *J* = 7.2, 4H, ArH), 7.20 (d, *J* = 7.6, 1H, ArH), 6.79 (d, *J* = 8.4, 1H, D_2O exchangeable, NH), 4.65–4.58 (m, 3H, $\text{CH}_2\text{CH}_2\text{CO}$ & NHCHCO), 4.30 (s, 2H, CH_2ph), 3.63 (s, 3H, OCH_3), 2.92 (t, *J* = 7.2, 2H, $\text{CH}_2\text{CH}_2\text{CO}$), 1.62–1.50 (m, 3H, $\text{CH}_2\text{CH}(\text{CH}_3)_2$), 0.87–0.83 (m, 6H, 2CH₃). ^{13}C NMR (101 MHz, CDCl_3) δ 173.43 (C=O), 170.21 (C=O), 159.53 (C=O) ring, 145.85 (C-Ar), 137.69 (C-Ar), 133.03 (CH-Ar), 131.33 (CH-Ar), 129.13 (C-Ar), 128.75 (2CH-Ar), 128.37 (2CH-Ar), 128.11 (C-Ar), 127.19 (CH-Ar), 126.78 (CH-Ar), 125.29 (CH-Ar), 52.14 (NHCH_2CO), 50.76 (OCH_3), 46.99 ($\text{CH}_2\text{CH}_2\text{CO}$), 41.33 (CH_2CH), 38.94 (CH_2ph), 35.33 ($\text{CH}_2\text{CH}_2\text{CO}$), 24.81 ($\text{CH}(\text{CH}_3)_2$), 22.74 (CH_3), 21.79 (CH_3). IR (KBr) cm^{-1} : 3306, 3065, 2973, 2920 (H-Al), 1749, 1737, 1641 (C=O) ester, 1581. MS (MALDI, positive mode, matrix DHB) m/z : 458.54 (M + Na)⁺. Elemental analysis calculated for $\text{C}_{25}\text{H}_{29}\text{N}_3\text{O}_4$ (436.0) C, 68.95; H, 6.71; N, 9.65 found: C, 68.90; H, 6.76; N, 9.60.

*Synthesis of hydrazides **9a–c**.* To a solution of ester **8a** (3.49 g, 0.01 mol) in ethyl alcohol (30 ml) was added hydrazine hydrate (0.5 ml, 0.01 mol). The reaction mixture was refluxed for 6 h, cooled and the white precipitate filtered and recrystallized from ethanol to obtain the corresponding hydrazide 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-(2-hydrazinyl-2-oxoethyl) propenamide (**9a**). By the same method, 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-(3-hydrazinyl-3-oxopropyl) propanamide (**9b**) and 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-(1-hydrazinyl-4-methyl-1-oxopentan-2-yl)propanamide (**9c**) can be prepared from reflux of the ester **8b** and **8c** (3.8042 g, 0.01 mol) and (4.3552 g, 0.01 mol) in ethyl alcohol (30 ml) with hydrazine hydrate (0.5 ml, 0.01 mol) for 6 h and then recrystallized from boiling ethanol.

*Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-(2-hydrazinyl-2-oxoethyl) propanamide (**9a**).* White crystals; yield (90%); mp: 170 °C; ^1H NMR (400 MHz, DMSO-d_6) (δ , ppm), (*J*, Hz): 9.07 (brs, 1H, D_2O exchangeable, NHNH_2), 8.28 (d, *J* = 8.4, 2H, ArH), 7.90 (d, *J* = 8, 1H, ArH), 7.83–7.76 (m, 2H, ArH), 7.34–7.25 (m, 4H, ArH), 7.19–7.15 (m, 1H, D_2O exchangeable, NH), 4.40–4.36 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}$), 4.30 (s, 2H, CH_2ph), 3.69–3.66 (m, 2H, NHCH_2CO), 3.40 (brs, 2H, D_2O exchangeable, NH₂), 2.72 (t, *J* = 7.6, 2H, $\text{CH}_2\text{CH}_2\text{CO}$). ^{13}C NMR (101 MHz, DMSO) δ 170.79 (C=O), 168.75 (C=O), 158.64 (C=O) ring, 145.58 (C-Ar), 138.58 (CH-Ar), 133.71 (CH-Ar), 132.11 (CH-Ar), 129.02 (C-Ar & 2CH-Ar), 128.84 (2CH-Ar), 127.98 (C-Ar), 126.96 (CH-Ar), 126.85 (C-Ar), 126.14 (CH-Ar), 47.28 ($\text{CH}_2\text{CH}_2\text{CO}$), 41.42 (NHCH_2CO), 38.22 (CH_2ph), 34.57 ($\text{CH}_2\text{CH}_2\text{CO}$). IR (KBr) cm^{-1} : 3445, 3292 (NH-NH₂), 3200 (NH-NH₂), 3059, 2947, 2935, 1720, 1634, 1620, 1581. MS (MALDI, positive mode, matrix DHB) m/z : 402.43 (M + Na)⁺. Elemental analysis calculated for $\text{C}_{20}\text{H}_{21}\text{N}_5\text{O}_3$ (379.4) C, 63.31; H, 5.58; N, 18.46 found: C, 63.34; H, 5.53; N, 18.41.

*Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-*N*-(3-hydrazinyl-3-oxopropyl) propanamide (**9b**).* White crystals; yield (88%); mp: 222 °C; ^1H NMR (400 MHz, DMSO-d_6) (δ , ppm), (*J*, Hz): 9.09–9.03 (m, 1H, D_2O exchangeable, NHNH_2), 8.26–8.10 (m, 2H, ArH), 7.88–7.77 (m, 3H, ArH), 7.33–7.23 (m, 4H, ArH), 7.17 (brs, 1H, D_2O exchangeable, NH), 4.35–4.33 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}$), 4.28 (s, 2H, CH_2ph), 3.43 (brs, 2H, D_2O exchangeable, NH₂), 2.63 (s, 2H), 2.63–2.60 (m, 2H, NHCH_2CH_2), 1.84–1.76 (m,



4H, 2CH₂CH₂CO). ¹³C NMR (101 MHz, DMSO) δ 170.26 (C=O), 170.20 (C=O), 158.56 (C=O) ring, 145.42 (C-Ar), 138.61 (C-Ar), 133.68 (CH-Ar), 132.08 (CH-Ar), 129.02 (C-Ar & 2CH-Ar), 128.83 (2CH-Ar), 128.03 (C-Ar), 126.97 (CH-Ar), 126.84 (CH-Ar), 126.14 (CH-Ar), 47.43 (CH₂CH₂CO), 38.24 (CH₂ph), 35.84 (NHCH₂-CH₂CO), 34.70 (NHCH₂CH₂CO), 34.07 (CH₂CH₂CO). IR (KBr) cm⁻¹: 3427, 3300 (NH-NH₂), 3210 (NH-NH₂), 3086, 2931, 2863, 1655, 1649, 1608, 1581. MS (MALDI, positive mode, matrix DHB) *m/z*: 416.46 (M + Na)⁺. Elemental analysis calculated for C₂₁H₂₃N₅O₃ (393.4) C, 64.11; H, 5.89; N, 17.80 found: C, 64.14; H, 5.85; N, 17.84.

Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(1-hydrazinyl-4-methyl-1-oxopentan-2-yl) propanamide (9c). White crystals; yield (89%); mp: 220 °C; ¹H NMR (400 MHz, DMSO-*d*₆) (δ , ppm), (*J*, Hz): 9.13 (brs, 1H, D₂O exchangeable, NHNH₂), 8.29–8.25 (m, 1H, ArH), 8.08–8.05 (m, 1H, ArH), 7.90–7.77 (m, 3H, ArH), 7.35–7.16 (m, 4H, ArH), 7.20–7.16 (m, 1H, D₂O exchangeable, NH), 4.45–4.37 (m, 3H, CH₂CH₂CO & NHCHCO), 4.30 (s, 2H, CH₂ph), 3.43 (brs, 2H, D₂O exchangeable, NH₂), 2.77–2.65 (m, 2H, CH₂CH₂CO), 1.43–1.39 (m, 3H, CH₂CH), 0.76 (d, *J* = 6.8, 6H, 2CH₃). ¹³C NMR (101 MHz, DMSO) δ 171.66 (C=O), 170.04 (C=O), 158.58 (C=O) ring, 145.45 (C-Ar), 138.59 (C-Ar), 133.64 (CH-Ar), 132.03 (CH-Ar), 129.01 (C-Ar & 2CH-Ar), 128.83 (2CH-Ar), 128.04 (C-Ar), 126.93 (CH-Ar), 126.85 (CH-Ar), 126.11 (CH-Ar), 50.17 (NHCHCO), 47.23 (CH₂CH₂CO), 41.57 (CH₂CH(CH₃)₂), 38.27 (CH₂ph), 34.58 (CH₂CH₂CO), 24.62 (CH₂CH(CH₃)₂), 23.25 (CH₃), 22.11 (CH₃). IR (KBr) cm⁻¹: 3414, 3292 (NH-NH₂), 3116 (NH-NH₂), 3065, 2959, 2922, 2867, 1732, 1645, 1604, 1585. MS (MALDI, positive mode, matrix DHB) *m/z*: 458.54 (M + Na)⁺. Elemental analysis calculated for C₂₄H₂₉N₅O₃ (435.5) C, 66.19; H, 6.71; N, 16.08 found: C, 66.14; H, 6.76; N, 16.03.

General procedure for synthesis of phthalazinone amino derivatives of glycine 11a–d. Under azide coupling conditions as previewed before, A cold solution at (−5 °C) of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-hydrazineyl-2-oxoethyl) propanamide (9a) (3.5338 g, 10 mmol) in acetic acid (60 ml) and hydrochloric acid (5N, 30 ml) was added portion wise under stirring to a cold solution (0 °C) of sodium nitrite (0.7 g, 0.01 mol) in water (30 ml). After stirring at the same temperature for 30 minutes, the *in situ* generated azide 10 was extracted with cold ethyl acetate and washed successively with cold water and 5% Na₂CO₃. After drying over anhydrous sodium sulphate, the azide 10 was used without further purification in the next step. Amines (12 mmol) were added to the previously prepared cold dried solution of the azide. Next, the combination was chilled for 24 hours before being left at room temperature for a further 24 hours. The products 11a–d were obtained by filtering the reaction mixture, washing the filtrate with 0.1N HCl, 5% Na₂CO₃, and water, and finally drying it on anhydrous sodium sulphate. The solvent was then evaporated under vacuum.

Synthesis of N-(2-(allylamino)-2-oxoethyl)-3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanamide (11a). Off-white crystals; yield (68%); mp: 165 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.38–8.35 (m, 1H, ArH), 7.71–7.64 (m, 3H, ArH), 7.39–7.36 (m, 1H, ArH), 7.29 (d, *J* = 7.2, 3H, ArH), 7.21–7.18 (m, 3H,

D₂O exchangeable, 2NH & ArH), 5.83–5.74 (m, 1H, CHCH₂), 5.15 (d, *J*_{trans} = 17.1, 1H, CHCH₂), 5.05 (d, *J*_{cis} = 10.4, 1H, CHCH₂), 4.58 (t, *J* = 6.7, 2H, CH₂CH₂CO), 4.28 (s, 2H, CH₂ph), 3.97–3.95 (m, 2H, NHCH₂CO), 3.86–3.83 (m, 2H, NHCH₂CH), 2.88 (t, *J* = 7.6, 2H, CH₂CH₂CO). ¹³C NMR (101 MHz, CDCl₃) δ 171.31 (C=O), 169.00 (C=O), 159.45 (C=O) ring, 145.82 (C-Ar), 137.69 (C-Ar), 133.96 (CHCH₂), 133.00 (CH-Ar), 131.30 (CH-Ar), 129.22 (C-Ar), 128.73 (2CH-Ar), 128.39 (2CH-Ar), 128.08 (C-Ar), 127.17 (CH-Ar), 126.77 (CH-Ar), 125.25 (CH-Ar), 116.20 (CHCH₂), 47.29 (CH₂CH₂CO), 43.59 (NHCH₂CO), 41.86 (CH₂CHCH₂), 38.88 (CH₂ph), 35.34 (CH₂CH₂CO). IR (KBr) cm⁻¹: 3300, 3086, 2969, (2920 & 2853) H-Al, 1720, 1645, 1630, 1579. MS (MALDI, positive mode, matrix DHB) *m/z*: 427.48 (M + Na)⁺. Elemental analysis calculated for C₂₃H₂₄N₄O₃ (404.5) C, 68.30; H, 5.98; N, 13.85 found: C, 68.35; H, 5.93; N, 13.80.

Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-(butylamino)-2-oxoethyl) propanamide (11b). Off-white crystals; yield (66%); mp: 161 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.41–8.36 (m, 1H, ArH), 7.72–7.66 (m, 3H, ArH), 7.29 (d, *J* = 8.0, 4H, ArH), 7.20–7.18 (m, 1H, ArH), 7.12–7.10 (m, 1H, D₂O exchangeable, NHCH₂), 6.92–6.88 (m, 1H, D₂O exchangeable, NHCH₂CH₂), 4.60–4.54 (m, 2H, CH₂CH₂CO), 4.28 (s, 2H, CH₂ph), 3.90 (s, 2H, NHCH₂CO), 3.25–3.17 (m, 2H, NHCH₂CH₂), 2.87 (t, *J* = 7.4, 2H, CH₂CH₂CO), 1.49–1.41 (m, 2H, CH₂CH₂CH₃), 1.36–1.27 (m, 2H, CH₂CH₃), 0.89 (t, *J* = 7.6, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 171.26 (C=O), 170.14 (C=O), 169.01 (C=O) ring, 145.83 (C-Ar), 137.68 (C-Ar), 133.02 (CH-Ar), 131.31 (CH-Ar), 129.22 (C-Ar), 128.72 (2CH-Ar), 128.38 (2CH-Ar), 128.08 (C-Ar), 127.13 (CH-Ar), 126.76 (CH-Ar), 125.26 (CH-Ar), 47.30 (CH₂CH₂CO), 43.56 (NHCH₂CO), 39.39 (NHCH₂), 38.88 (CH₂ph), 35.32 (CH₂CH₂CO), 31.64 (CH₂CH₂CH₃), 20.03 (CH₂CH₂CH₃), 13.67 (CH₃). IR (KBr) cm⁻¹: 3288, 3090, 2985, 2931, 2873 (H-Al), 1728, 1645, 1626, 1583. MS (MALDI, positive mode, matrix DHB) *m/z*: 443.52 (M + Na)⁺. Elemental analysis calculated for C₂₄H₂₈N₄O₃ (420.5) C, 68.55; H, 6.71; N, 13.32 found: C, 68.50; H, 6.76; N, 13.37.

Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-oxo-2-(piperidin-1-yl) ethyl) propanamide (11c). Off-white crystals; yield (61%); mp: 110 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.44–8.42 (m, 1H, ArH), 7.71–7.65 (m, 3H, ArH), 7.29–7.23 (m, 3H, ArH), 7.21–7.17 (m, 1H, ArH), 7.05 (brs, 1H, D₂O exchangeable, NH), 4.60 (t, *J* = 7.6, 2H, CH₂CH₂CO), 4.30 (s, 2H, CH₂ph), 4.03 (s, 2H, NHCH₂CO), 3.55–3.52 (m, 2H, NCH₂), 3.31–3.28 (m, 2H, NCH₂), 2.87 (t, *J* = 7.4, 2H, CH₂CH₂CO), 1.63 (q, *J* = 5.6, 2H, CH₂CH₂CH₂), 1.53 (t, *J* = 5.6, 4H, CH₂CH₂CH₂). ¹³C NMR (101 MHz, CDCl₃) δ 170.46 (C=O), 165.99 (C=O), 159.23 (C=O) ring, 145.36 (C-Ar), 137.94 (CH-Ar), 132.79 (CH-Ar), 131.12 (CH-Ar), 129.23 (C-Ar), 128.66 (2CH-Ar), 128.36 (2CH-Ar), 128.35 (C-Ar), 127.24 (CH-Ar), 126.63 (CH-Ar), 125.15 (CH-Ar), 47.22 (CH₂CH₂CO), 45.42 (NCH₂CH₂), 43.11 (NCH₂CH₂), 41.31 (NHCH₂CO), 38.88 (CH₂ph), 35.04 (CH₂CH₂CO), 26.11 (CH₂CH₂CH₂), 25.36 (CH₂CH₂CH₂), 24.31 (CH₂CH₂CH₂). IR (KBr) cm⁻¹: 3308, 3059, 2933, 2853 (H-Al), 1730, 1657, 1630, 1581. MS (MALDI, positive mode, matrix DHB) *m/z*: 455.53 (M + Na)⁺. Elemental analysis calculated for C₂₅H₂₈N₄O₃ (432.5) C, 69.42; H, 6.53; N, 12.95 found: C, 69.47; H, 6.58; N, 12.90.



Synthesis of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-morpholino-2-oxoethyl) propanamide (11d). Off-white crystals; yield (62%); mp: 123 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.39–8.37 (m, 1H, ArH), 7.69–7.62 (m, 3H, ArH), 7.26–7.22 (m, 5H, ArH), 7.18–7.14 (m, 1H, D₂O exchangeable, NH), 4.56 (t, *J* = 7.2, 2H, CH₂CH₂CO), 4.26 (s, 2H, CH₂-ph), 4.03–4.02 (m, 2H, NHCH₂CO), 3.61–3.59 (m, 4H, 2CH₂O), 3.55–3.52 (m, 2H, NCH₂), 3.35–3.33 (m, 2H, NCH₂), 2.87 (t, *J* = 7.6, 2H, CH₂CH₂CO). ¹³C NMR (101 MHz, CDCl₃) δ 170.71 (C=O), 166.79 (C=O), 159.24 (C=O) ring, 145.45 (C-Ar), 137.90 (C-Ar), 132.85 (CH-Ar), 131.19 (CH-Ar), 129.18 (C-Ar), 128.67 (2CH-Ar), 128.35 (2CH-Ar), 127.72 (C-Ar), 127.17 (CH-Ar), 126.65 (CH-Ar), 125.18 (CH-Ar), 66.58 (CH₂O), 66.27 (CH₂O), 47.25 (CH₂CH₂CO), 44.85 (NHCH₂CH₂O), 42.24 (NHCH₂CO), 41.17 (NHCH₂CO), 38.82 (CH₂ph), 34.99 (CH₂CH₂CO). IR (KBr) cm⁻¹: 3308, 3057, 2963, (2931 & 2855) H-Al, 1732, 1663, 1636, 1581. MS (MALDI, positive mode, matrix DHB) *m/z*: 457.52 (M + Na)⁺. Elemental analysis calculated for C₂₄H₂₆N₄O₄ (434.5) C, 66.34; H, 6.03; N, 12.89 found: C, 66.39; H, 6.08; N, 12.86.

General procedure for preparation methyl (3-[4-benzyl-1-oxophthalazin-2(1H)-yl] propylamine)alkanoates 12a–c. A cold solution of 3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-hydrazinyl-2-oxoethyl) propanamide (9a) (3.53 g, 10 mmol) at (−5 °C) in acetic acid (60 ml) and hydrochloric acid (5N, 30 ml) was added portion wise under stirring to a cold solution (0 °C) of sodium nitrite (0.7 g, 0.01 mol) in water (30 ml). After stirring at the same temperature for 30 minutes, the *in situ* generated azide 10 was extracted with cold ethyl acetate and washed successively with cold water and 5% Na₂CO₃. After drying over anhydrous sodium sulphate, the azide 10 was used without further purification in the next step.

After combining “glycine, β-Alanine, and L-Leucine” with triethyl amine (1 g, 10 mmol) in an ethyl acetate solution at −5 °C for 15 minutes, the amino acid methyl ester hydrochloride solution was added to the azide cold dried solution that had been previously made. The next step was to chill the combination for 24 hours before letting it sit at room temperature for another hour. Products 12a–c were obtained by filtering the reaction mixture, washing the resulting solution with 0.1N HCl, 5% Na₂CO₃, and water, and finally drying it over anhydrous sodium sulfate. The solvent was subsequently evaporated under vacuum.

Synthesis of methyl (3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoyl) glycylglycinate (12a). White crystals; yield (72%); mp: 178 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.35–8.33 (m, 1H, ArH), 7.71–7.64 (m, 3H, ArH), 7.59–7.58 (m, 1H, ArH), 7.45 (brs, 1H, D₂O exchangeable, NH), 7.26 (d, *J* = 7.2, 4H, ArH), 7.19 (brs, 1H, D₂O exchangeable, NH), 4.58 (t, *J* = 6.8, 2H, CH₂CH₂CO), 4.28 (s, 2H, CH₂-ph), 4.02–3.99 (m, 4H, 2NHCH₂CO), 3.67 (s, 3H, OCH₃), 2.89 (t, *J* = 7.2, 2H, CH₂CH₂CO). ¹³C NMR (101 MHz, CDCl₃) δ 171.41 (C=O), 170.21 (C=O), 169.73 (C=O), 159.48 (C=O) ring, 145.87 (C-Ar), 137.70 (C-Ar), 132.97 (CH-Ar), 131.29 (CH-Ar), 129.22 (C-Ar), 128.71 (2CH-Ar), 128.39 (2CH-Ar), 128.06 (C-Ar), 127.09 (CH-Ar), 126.75 (CH-Ar), 125.25 (CH-Ar), 52.17 (OCH₃), 47.36 (CH₂CH₂CO), 43.26 (NHCH₂CO), 41.14 (NHCH₂COO), 38.85 (CH₂ph), 35.33 (CH₂CH₂CO). IR

(KBr) cm⁻¹: 3388, 3314, 3071, 2945, 2861 (H-Al), 1761, 1744, 1665, 1637 (C=O) ester. MS (MALDI, positive mode, matrix DHB) *m/z*: 459.48 (M + Na)⁺. Elemental analysis calculated for C₂₃H₂₄N₄O₅ (436.5) C, 63.29; H, 5.54; N, 12.84 found: C, 63.25; H, 5.59; N, 12.89.

Synthesis of methyl 3-(2-(3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanamido) acetamido) propanoate (12b). Off-white crystals; yield (70%) mp: 120 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.40–8.37 (m, 1H, ArH), 7.74–7.65 (m, 4H, ArH), 7.27 (d, *J* = 8, 4H, ArH), 7.21–7.17 (m, 2H, D₂O exchangeable, 2NH), 4.58 (t, *J* = 7.6, 2H, CH₂CH₂CO), 4.29 (s, 2H, CH₂-ph), 3.91–3.89 (m, 2H, NHCH₂CO), 3.65 (s, 3H, OCH₃), 3.53–3.47 (m, 2H, NHCH₂CH₂), 2.88 (t, *J* = 7.6, 2H, CH₂CH₂CO), 2.55–2.51 (m, 2H, NHCH₂CH₂). ¹³C NMR (101 MHz, CDCl₃) δ 172.58 (C=O), 171.24 (C=O), 169.21 (C=O), 159.45 (C=O) ring, 145.87 (C-Ar), 137.70 (C-Ar), 133.04 (CH-Ar), 131.37 (CH-Ar), 129.18 (C-Ar), 128.74 (2CH-Ar), 128.38 (2CH-Ar), 128.04 (C-Ar), 127.11 (CH-Ar), 126.78 (CH-Ar), 125.29 (CH-Ar), 51.79 (OCH₃), 47.34 (CH₂CH₂CO), 43.37 (NHCH₂CO), 38.88 (CH₂ph), 35.30 (CH₂CH₂CO), 35.09 (NHCH₂CH₂CO), 33.78 (NHCH₂CH₂CO), 29.03 (NHCH₂CH₂), Dept 135 (101 MHz, CDCl₃) δ 132.97 (CH-Ar), 131.31 (CH-Ar), 128.71 (2CH-Ar), 128.38 (2CH-Ar), 127.13 (CH-Ar), 126.75 (CH-Ar), 125.24 (CH-Ar), 51.70 (OCH₃), 47.33 (CH₂CH₂CO), 43.46 (NHCH₂CO), 38.93 (CH₂-ph), 35.34 (CH₂CH₂CO), 35.12 (NHCH₂CH₂CO), 33.82 (NHCH₂CH₂CO). IR (KBr) cm⁻¹: 3296, 3086, 3065, 2955, 2849 (H-Al), 1724, 1734, 1643, 1624 (C=O) ester, 1583. MS (MALDI, positive mode, matrix DHB) *m/z*: 473.51 (M + Na)⁺. Elemental analysis calculated for C₂₄H₂₆N₄O₅ (450.5) C, 63.99; H, 5.82; N, 12.44 found: C, 63.95; H, 5.87; N, 12.40.

Synthesis of methyl (3-(4-benzyl-1-oxophthalazin-2(1H)-yl) propanoyl) glycylleucinate (12c). Off-white crystals; yield (73%); mp: 125 °C; ¹H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): 8.37–8.35 (m, 1H, ArH), 7.70–7.63 (m, 3H, ArH), 7.44–7.42 (m, 2H, D₂O exchangeable, ArH & NH), 7.28–7.24 (m, 4H, ArH), 7.18–7.15 (m, 1H, D₂O exchangeable, NH), 4.56 (t, *J* = 7.1, 2H, CH₂CH₂CO), 4.26 (s, 2H, CH₂-ph), 4.12–3.93 (m, 3H, NHCH₂CO & NHCHCO), 3.66 (s, 3H, OCH₃), 2.87 (t, *J* = 7.2, 2H, CH₂CH₂CO), 1.61 (d, *J* = 6.8, 2H, CH₂CH), 1.24 (t, *J* = 6.4, 1H, CH₂CH), 0.92–0.87 (m, 6H, 2CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 173.20 (C=O), 171.36 (C=O), 169.22 (C=O), 159.35 (C=O) ring, 145.78 (C-Ar), 137.72 (C-Ar), 132.94 (CH-Ar), 131.25 (CH-Ar), 129.20 (C-Ar), 128.69 (2CH-Ar), 128.38 (2CH-Ar), 128.09 (C-Ar), 127.15 (CH-Ar), 126.72 (CH-Ar), 125.21 (CH-Ar), 52.16 (NHCHCO), 50.92 (OCH₃), 47.38 (CH₂CH₂CO), 43.27 (NHCH₂CO), 41.01 (CH₂CH(CH₃)₂), 38.83 (CH₂ph), 35.13 (CH₂CH₂CO), 24.80 (CH(CH₃)₂), 22.74 (CH₃), 21.78 (CH₃). IR (KBr) cm⁻¹: 3300, 3220, 3063, 2961, 2867 (H-Al), 1741, 1730, 1645, 1628 (C=O) ester, 1583. MS (MALDI, positive mode, matrix DHB) *m/z*: 515.59 (M + Na)⁺. Elemental analysis calculated for C₂₇H₃₂N₄O₅ (492.6) C, 65.84; H, 6.55; N, 11.37 found: C, 65.84; H, 6.55; N, 11.37.

Synthesis of (Z)-3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-(4-nitrobenzylidene) hydrazinyl)-2-oxoethyl)propanamide (13a). A mixture of hydrazide 9a (3.53 g, 0.01 mol) and 4-nitrobenzaldehyde (3.02 g, 0.02 mol) in ethanol (30 ml) was refluxed for 24 h. By cooling the solid product formed, filtered off and recrystallized from ethanol solvent gave compound 13a.



Off-white crystals; yield (80%); mp: 240 °C; ^1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): δ = (Z/E isomers mixture 78/22) 11.71 & 11.68 (2 s, 1H, D_2O exchangeable, CONHN), 8.09 & 8.41 (2 s, 1H, N=CH), 8.34–8.25 (m, 4H, ArH), 7.96–7.81 (m, 5H, ArH), 7.36 (d, *J* = 7.7, 2H, ArH), 7.28 (t, *J* = 8, 3H, ArH), 7.18 (s, 1H, D_2O exchangeable, CONHCH₂), 4.42–4.38 (m, 2H, CH_2 -CH₂CO), 4.32 (s, 2H, CH₂-ph), 4.29 (s, 1H, NHCH₂CO), 3.86 (s, 1H, NHCH₂CO), 2.77 (s, 2H, CH₂CH₂CO). IR (KBr) cm⁻¹: 3431, 3355, 3208 (H-Ar), 3114 (H-Ar), 3087, 2957, 2925, 2851, 1739, 1694, 1622, 1524 (NO₂), 1597 (C=N). MS (MALDI, positive mode, matrix DHB) *m/z*: 535.55 (M + Na)⁺. Elemental analysis calculated for C₂₇H₂₄N₆O₅ (512.5) C, 63.27; H, 4.72; N, 16.40 found: C, 63.22; H, 4.77; N, 16.45.

Synthesis of (Z)-3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-(4-chlorobenzylidene) hydrazinyl)-2-oxoethylpropanamide (13b). A mixture of hydrazide **9a** (3.53 g, 0.01 mol) and *p*-chlorobenzaldehyde (2.81 g, 0.02 mol) in ethanol (30 ml) was refluxed for 24 h. By cooling the solid product formed, filtered off and recrystallized from ethanol solvent gave compound **13b**.

Off-white crystals; yield (85%); mp: 190 °C; ^1H NMR (400 MHz, chloroform-*d*) (δ , ppm), (*J*, Hz): δ = (Z/E isomers mixture 80/20) 10.17 & 10.55 (2 s, 1H, D_2O exchangeable, CONHN), 8.44–8.34 (m, 1H, ArH), 7.94 & 8.18 (2 s, 1H, N=CH), 7.76–7.70 (m, 3H, ArH), 7.64–7.61 (m, 2H, ArH), 7.40–7.37 (m, 3H, ArH), 7.28–7.26 (m, 4H, ArH), 7.19 & 7.45 (2 s, 1H, D_2O exchangeable, CONHCH₂), 4.69–4.64 (m, 2H, CH₂CH₂CO), 4.54–4.52 (m, 2H, NHCH₂CO), 4.33 & 4.05 (2 s, 2H, CH₂-ph), 3.01–2.90 (m, 2H, CH₂CH₂CO). IR (KBr) cm⁻¹: 3406, 3296, (3214 & 3132) H-Ar, 3064, 2962, 2927, 1688, 1643, 1632, 1581. MS (MALDI, positive mode, matrix DHB) *m/z*: 524.99 (M + Na)⁺. Elemental analysis calculated for C₂₇H₂₄ClN₅O₃ (502.0) C, 64.60; H, 4.82; Cl, 7.06; N, 13.95 found: C, 64.65; H, 4.87; Cl, 7.04; N, 13.90.

Synthesis of (Z)-3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-(4-methoxybenzylidene) hydrazinyl)-2-oxoethylpropanamide (13c). A mixture of hydrazide **9a** (3.53 g, 0.01 mol) and 4-methoxybenzaldehyde (2.72 g, 0.02 mol) in ethanol (30 ml) was refluxed for 24 h. By cooling the solid product formed, filtered off and recrystallized from ethanol solvent gave compound **13c**.

Off-white crystals; yield (89%); mp: 191 °C; ^1H NMR (400 MHz, DMSO-*d*₆) (δ , ppm), (*J*, Hz): δ = (Z/E isomers mixture 75/25) 11.29 & 11.23 (2 s, 1H, D_2O exchangeable, CONHN), 8.29 & 8.38 (2 s, 1H, N=CH), 8.21–8.18 (m, 1H, ArH), 7.94–7.80 (m, 2H, ArH), 7.86–7.80 (m, 2H, ArH), 7.64–7.59 (m, 2H, ArH), 7.37–7.27 (m, 4H, ArH), 7.18 & 7.09 (2 s, 1H, D_2O exchangeable, CONHCH₂), 4.43–4.39 (m, 2H, CH₂CH₂CO), 4.31 (s, 2H, CH₂-ph), 4.24 (s, 2H, NHCH₂CO), 3.80 (s, 3H, OCH₃), 2.79–2.75 (m, 2H, CH₂CH₂CO). MS (MALDI, positive mode, matrix DHB) *m/z*: 520.58 (M + Na)⁺. Elemental analysis calculated for C₂₈H₂₇N₅O₄ (497.6) C, 67.59; H, 5.47; N, 14.08 found: C, 67.54; H, 5.45; N, 14.04.

Synthesis of (Z)-3-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(2-(5-bromo-2-hydroxybenzylidene) hydrazinyl)-2-oxoethylpropanamide (13d). A solution containing 3.53 g of hydrazide **9a** and 0.01 mol of 5-bromo-2-hydroxybenzaldehyde in 30 ml of ethanol was refluxed for 24 hours. Compound **13d** was obtained by chilling the solid product, filtering it out, and then recrystallizing it from the ethanol solvent.

Off-white crystals; yield (88%); mp: 130 °C; ^1H NMR (400 MHz, DMSO-*d*₆) (δ , ppm), (*J*, Hz): δ = (Z/E isomers mixture 50/50) 11.69 & 11.44 (2 s, 1H, D_2O exchangeable, CONHN), 8.41 & 8.35 (2 s, 1H, N=CH), 8.30–8.25 (m, 2H, ArH), 8.22 (s, 2H, ArH), 7.91 (d, *J* = 7.8, 2H, ArH), 7.85–7.76 (m, 4H, ArH), 7.35 (d, *J* = 7.8, 2H, ArH), 7.30–7.26 (m, 3H, ArH), 7.20–7.17 (m, 1H, D_2O exchangeable, NHCH₂CO), 4.43–4.36 (m, 2H, CH₂CH₂CO), 4.31 (s, 2H, CH₂-ph), 4.31 (s, 2H, NHCH₂CO), 4.24 (s, 1H, D_2O exchangeable, OH), 2.77–3.70 (m, 2H, CH₂CH₂CO). IR (KBr) cm⁻¹: 3433, 3286, 3212 (H-Ar), 3067, 2963, 2920, 1677, 1649, 1624, 1577. MS (MALDI, positive mode, matrix DHB) *m/z*: 585.44 (M + Na)⁺. Elemental analysis calculated for C₂₇H₂₄BrN₅O₄ (562.4) C, 57.66; H, 4.30; Br, 14.21; N, 12.45 found: C, 57.63; H, 4.35; Br, 14.22; N, 12.40.

Biological assays

Cytotoxicity of the synthesized compounds using MTT assay

HCT-116 cancer and normal liver WI-38 cell lines were cultured in complete media of “DMEM at 5% carbon dioxide and 37 °C” following standard tissue culture work. The cells were grown in “10% fetal bovine serum (FBS) and 1% penicillin-streptomycin” in 96-multiwell plate. The synthesized compounds were screened for their cytotoxicity using 20 μL of MTT solution (Promega, USA) for 48 hours^{25,26} with concentrations of “0.01, 0.1, 1, 10, and 100 μM ” for 48 h. The plate was cultured for 3 hours. Percentage of cell viability was calculated following this equation (100 – (A_{sample})/(A_{control})) × 100.²⁷

VEGFR inhibition

The most promising cytotoxic compounds were subjected to VEGFR2 Kinase Assay Kit Catalog #40325 using ELISA kit ELISA Assay following manufacturer information.²⁸ The luminescence was measured with a microplate reader at 450 nm by ELISA Reader (PerkinElmer). The inhibition percentage was calculated following this equation: 100 – $\left[\frac{A_{\text{control}}}{A_{\text{treated}}} - \text{control} \right]$, IC₅₀ was determined using GraphPad prism7.

Flow cytometry using annexin V/PI staining

After a night of incubation in 6-well culture plates with 3–5 × 10⁵ cells per well, compound **7d** was added to the cells and left to treat for 48 hours according to the IC₅₀ values. After that, the cells were incubated in a 100 μL solution of Annexin binding buffer “25 mM CaCl₂, 1.4 M NaCl, and 0.1 M Hepes/NaOH, pH 7.4” in the dark for 30 minutes with “Annexin V-FITC solution (1 : 100) and propidium iodide (PI) at a concentration equivalent to 10 g ml⁻¹”. The labeled cells were then extracted using the Cytoflex FACS machine.^{29,30}

Molecular docking study

Utilizing Maestro, protein, and compound structures were created and optimized. Binding sites inside proteins were then identified using the grid-box dimensions surrounding the co-crystallized ligands. Compounds were docked against the protein structures of VEGFR2 (PDB = 4ASD) using AutoDock



Vina software following routine work.^{31,32} Maestro was utilized to optimize protein and ligand structures. In terms of binding energy and ligand-receptor interactions, binding activities evaluated the results of molecular docking. Chimera-UCSF was then used to complete the visualization.

Conclusion

In conclusion, twenty-six new phthalazine derivatives were designed and synthesized beginning with 2-(4-benzyl-1-oxophthalazin-2(1H)-yl)-N-(3-hydrazinyl-3-oxo propyl) acetamide (2) by chemoselective *N*-alkylation *via* Michael addition reaction and their structures were interpreted by several analytical and spectroscopic techniques. Interestingly, compound 7d exhibited potent cytotoxicity with an IC₅₀ value 0.38 μM compared to Sorafenib (IC₅₀ = 2.93 μM). Compounds 7d exhibited potent VEGFR2 inhibition by 97.6% with an IC₅₀ value 21.9 μM compared to Sorafenib (94.7% and IC₅₀ of 30.1 μM). For apoptosis activity, 7d-treatment induced apoptosis by 23.6-fold, arresting the cell proliferation at G1-phase. Finally, it formed a good binding affinity towards VEGFR2 protein with a binding energy of -26.8 kcal mol⁻¹, and it formed binding interactions with the key interactive amino acids. Hence, compound 7d was worthy of studying as a target-oriented anti-liver agent with a good selectivity profile.

Author contributions

D. E. S., S. M. R., H. A. S. synthesized the entire series of derivatives with the characterization of structure elucidation. At the same time, I. E. A., M. S. A., A. H. K., and M. A. A. participated in characterization, data analysis, resources, and revision, while M. S. Nafie initiated the idea and design of the biology part by carrying out *in vitro* cytotoxic screening, flow cytometry, and *in silico* studies with the linguistic revision and manuscript finalizing. D. E. S., S. M. R., and M. S. Nafie wrote the original draft with the literature review in their corresponding parts. All authors agreed on the manuscript in the final submitted form.

Conflicts of interest

The authors declare no conflict of interest.

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