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Design, Synthesis and Evaluation of 1,3,2 Diazaphosphorin[4,5-b]Quinoxaline-5,10-di-N-oxide Derivatives as Novel VEGFR-2 and SRC Kinase Inhibitors in the Treatment of Prostate Cancer

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Abstract: Prostate cancer remains a significant public health problem, with limited therapeutic options. Recently, new approaches in prostate cancer treatment have been developed, inhibition of the proangiogenic VEGFR kinase and SRC kinase, involved in progression and metastasis of prostate cancer were investigated. A new series of 1,3,2diazaphosphorin[4,5-b]quinoxaline-5,10-di-N-oxide was synthesized using Lawson's reagent in a reaction that provides a rare example of the incorporation of phosphorous. The structure of the new compounds was confirmed by spectroscopic data. The new synthesized compounds were evaluated for their *in-vitro* prostate cancer cell line (PC3). Compounds **7a** and **8a** possessed remarkable antitumor activity more active than the known drug Doxorubicin with IC50= 0.12, 0.36 and 0.63 μM respectively. Evaluation of the new synthesized compounds on VEGFR-2 and SRC kinases showed that compound **7a** was the most effective with IC50= 18μM. On the other hand, weak inhibitory effect was observed by the compounds on SRC kinase. Docking study was performed for compound **7a** into ATP binding site of VEGFR-2 kinase.

Keywords: Diazaphosphorin, quinoxaline 1,4-di-N-oxides, prostate cancer, VEGFR, SRC kinase, molecular modeling.

1. INTRODUCTION

Prostate cancer remains a significant public health problem, with limited therapeutic options in the setting of castrate-resistant metastatic disease [1, 2]. Until recently, there were only four FDA-approved chemotherapeutic agents (estramustine [3], itoxantrone [4, 5], docetaxel [3, 5], cabazitaxel [6-9] with docetaxel and prednisone being a standard for patients requiring chemotherapy. However, the responses are not durable and the disease ultimately progresses [10]. A direct strategy depending on targeting tumor cells with chemotherapeutic agents has dominated the field of cancer therapy for several years [11] however, this strategy was also toxic to the non-malignant cells and would eventually develop resistance [12]. Recently, there was an improvement in cancer therapies aimed at the underlying cancer pathology [13]. One of these indirect anti-cancer therapies was based on the fact that tumor growth beyond a few millimeters and metastatic dissemination within the host depended on the induction of angiogenesis, or the process of new blood vessel formation [14-18]. One of the most important pro-angiogenic factors involved in tumor angiogenesis is vascular endothelial growth factor (VEGF) [15, 19]. Hence, blockade of the VEGF/VEGFR-signaling is regarded as an attractive therapeutic target for inhibition of tumor angiogenesis [13, 20]. In addition, several reports have implicated VEGF in prostate carcinogenesis and in its metastatic spread [21-26].Clinical trials, phase II and III, investigating VEGF-targeting therapy in combination with chemotherapeutic agents in prostate cancer treatment concluded that this combination is reasonably safe and effective [26-28]. Moreover, quinoxaline 1,4-di-N-oxides derivatives proved to be potential cytotoxic agents against prostate cancer [29-31], two novel quinoxaline 1,4-di-N-oxides derivatives Q39 [32] and TX-2098 [33], Fig. (**1**), were reported to exert their activity possibly through VEGF inhibition. Furthermore, the lead compound of this series of compounds, tirapazamine, has been successfully used in combination with low-dose metronomic cyclophosphamide in treatment of human prostate cancer PC-3 [34]. On the other hand, Src is an example of non-receptor TKs [35] involved in multiple signaling pathways central to prostate cancer development, progression and metastasis in addition to its osteoclast activities [36, 37] leading to increased VEGF expression [38]. As such, Src inhibition represents a valid therapeutic strategy for the treatment of advanced metastatic prostate cancer. In light of this data, it seemed clear that there is a great need to exploit novel targets and new agents in the search for more effective treatment [39-41]. The reported inhibitory effect of quinoxaline 1,4-di-N -oxide on VEGF expression [32, 33] combined with the cytotoxic activity of compounds derived from the 1,3,2-diazaphosphorine [42], encouraged us to synthesize and evaluate the inhibitory effect of a new series of 1,3,2-diazaphosphorine[4,5-b]quinoxaline5,10-di-N oxides against VEGFR-2 and Src kinases.

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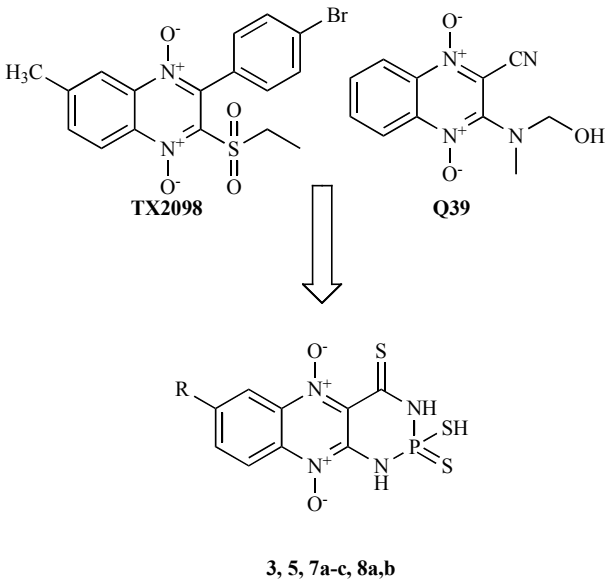


Fig. (1). New design of the synthesized compounds.

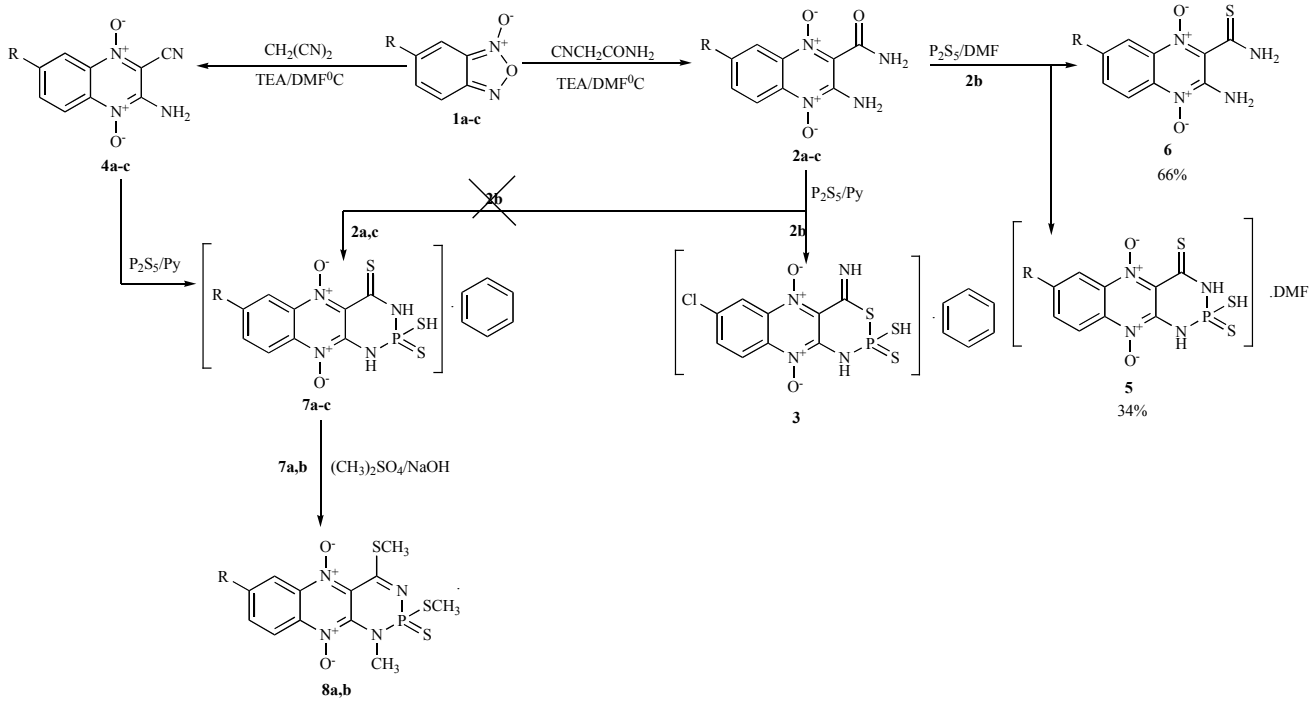
2. RESULTS AND DISCUSSION

2.1. Chemistry

Attempts to convert carbamoyl or carbonitrile groups using phosphorous pentasulphide in pyridine by previous research groups, proved that the reaction did not stop at the thionation step but rather proceeded all the way to the formation of annelated heterocyles and 1,3,2-diazaphosphorin rings [43-47]. This reaction provides a rare example of the incorporation of phosphorous derived from P_2S_5, into a heterocyclic system. Scheme **1** outlines a new synthetic pathway to prepare the tricyclic diazaphosphorin compounds **3**, **5**, **7a-c** and **8a, b** two key substrates were prepared, 2-amino-3-carbamoyl-6-substituted quinoxaline1,4-dioxide **2a-c** and 3-amino-7-substituted-2-quinoxalinecarbonitr-ile1,4 di-N-oxides **4a-c** [48].

They were obtained by the well-known Beirut [49, 50] reaction between 5-substitutedbenzo [c] [1, 2, 5]oxadiazol-1-oxides (benzofuroxanes,**1a,b**) and cyanoacetamide for derivatives **2a-c** or malononitrile for **4a-c**.The reaction of either 3-carbamoyl derivatives **2a,c** or 2-carbonitrile derivatives **4a-c** with P_2S_5 in pyridine yielded 2-mercapto-7-substituted [1, 3, 2] diazaphosphorin[4, 5-b]quinoxaline-5,10-di-N-oxide-2,4(1H,3H)-dithione in the form of pyridine solvates **7a-c**, Scheme **1**.While, compound **2b,** under the same reaction conditions, gave the azathiaphosphorine derivative **3.** The synthesized compounds were assigned their structures based on the spectral and microanalytical data. Infrared spectrum of the products **3, 7a-c** (isolated as pyridine solvates) was characterized by the disappearance of bands corresponding to cyano and carboxamide groups in addition to the bands at cm^{-1}3364-3231(2 NH); 1544, 1484 (NO); 1228(C=S). In the ^{1}H NMR spectra, the appearance of new signals corresponding to pyridine protons characterized the spectra in addition to the typical pattern of proton signals of quinoxaline-1,4-di-N-oxide, a broad NH signal was also observed. An SH proton signal is not clearly seen in the ^{1}H NMR spectra, which is consistent with the data concerning a study of the spectra for such compounds [44]. When this reaction was carried out in DMF, two products were obtained, the diazphosphorine **5** and in a larger portion 2-amino-3-carbamothioyl-6-chloroquinoxaline 1,4-dioxide **6**, proved by spectral and microanalytical data.

Furthermore, the removal of the pyridine in ether and methylation of the aqueous solution with dimethyl sulphate gave the trimethyl derivatives **7a,b** compound **7c** was excluded from this reaction due to its low yield. The ^{1}H NMR spectra of these compounds displayed singlets in the range 2.07-3.28 ppm corresponding to the methyl groups.



Scheme (1). Synthetic route for the preparation of the newly synthesizedcompounds.

A pluasible mechanism is presented in Fig. (**2**), the first step comprises acylation of the primary amino group by the P_2S_5, followed by the cyclization of this intermediate with concomitant evolution of H_2S gas.

2.2. Biological Studies

2.2.1. Cytotoxicity Studies

The new synthesized compounds were subjected to preliminary cytotoxic evaluation on human prostate cancer PC-3 using SRB method as previously described [51, 52]. The results of the tested compounds are presented in Table **1**. The best results obtained were by the pyridine solvates**7a** and **8a**, compared to doxorubicin(IC50= 0.12, 0.36 and 0.63μM, respectively). This shows that these new compounds were highly active cytotoxins to the human prostate cancer**,** it could be also suggested that higher cytotoxic activity were exerted by the 1,3,2-diazaphosphorin [4, 5-b] quinoxaline-1, 4-di-N-oxides derivatives unsubstituted in position 7. The two derivatives **5** and**7c**also showed good activity, IC50 values 0.5 and 0.75 μM, respectively. These results show that the pyridine solvates were better for the activity as compound **7a** and **7b** were almost four times more potent than **5**. On the other hand, methylation of the amino and thiol groups reduced the cytotoxic effect of compound **7a** by 3 folds while, compound **7b** has also expressed a one fold decrease in its activity.

2.2.2. Enzyme Inhibition Assay

In this investigation, a new series of 1,3,2-diazaphosphorine [4, 5-b] quinoxaline 1, 4-di-N oxides were synthesized and their ability to inhibit VEGFR-2 and Src tyrosine kinases were evaluated according to a radioisotope

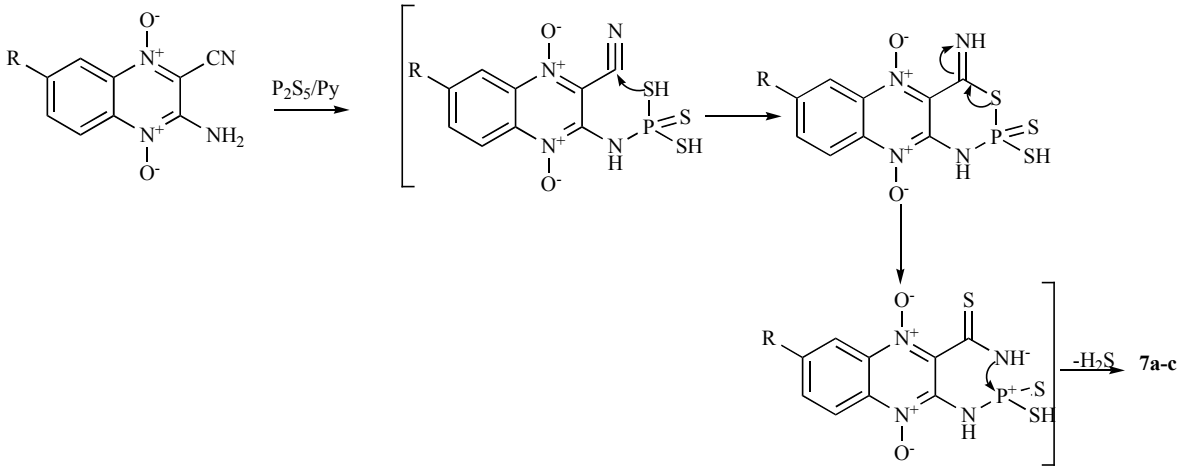
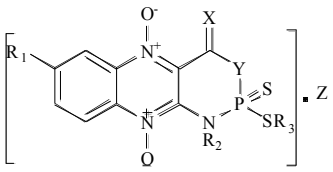


Fig. (2). Suggested mechanism for formation of 1,3,2-diazaphosphorin-2,4-dithione from P_2S_5.

Table 1. ***In-vitro*** **IC50 Values of the Target Compounds Assayed Against Human Prostate Cell Line PC-3**



Compound	R_1	R_2	R_3	X	Y	Z	IC50 PC-3 (μM)
3	Cl	H	H	NH	S		2.0
5	Cl	H	H	S	NH	DMF	0.5
7a	H	H	H	S	NH		0.12
7b	Cl	H	H	S	NH		2.13
7c	OCH_3	H	H	S	NH		0.75
8a	H	CH_3	CH_3	S	NCH_3	-	0.36
8b	Cl	CH_3	CH_3	S	NCH_3	-	3.13
Doxorubicin	-	-	-	-	-	-	0.63

Table 2. % Activity Change of SRC and VEGFR-2 Kinases Tested Against the Target Compounds

Compound	Src Kinase			VEGFR Kinase		
	% Activity Change 5 µM	% Activity Change 20 µM	% Activity Change 50 µM	% Activity Change 5 µM	% Activity Change 20 µM	% Activity Change 50 µM
3	2	11	-27	-5	-23	-43
5	-8	-5	-21	-6	-26	-58
7a	-7	-20	-17	-23	-54	-71
7b	-4	-8	-18	0	9	-17
7c	6	3	-10	-8	-22	-41
8a	5	2	-19	-1	-21	-35
8b	-6	-25	-31	-1	-14	-12

Table 3. IC50 Values of the Tested Compounds Against VEGFR-2 and Src Kinases

Compound	IC50 VEGFR-2 Kinase µM	IC50Src Kinase µM
3	64.11	ND
5	40.32	209.4
7a	17.8	3133
7b	ND[a]	303.9
7c	74.69	ND
8a	86.25	ND
8b	1587	142.8

[a]ND denotes the compounds where the IC50 could not be determined.

assay format for profiling evaluation of protein kinase targets. The results observed as percentage activity change compared to control are presented in Table **2**. The intra-assay variability was determined to be less than 10%. Inhibition of target activity by the compound gives negative values, while activation of target activity gives positive values. It has been considered that only values of>15% change in activity compared to control to be significant. The profiling data for the compounds against Src and VEGFR-2 target is presented in Table **2**. The profiling data for the compounds against VEGFR-2 target ranged from good to weak inhibition of its activity, Table **2**. The 2-mercapto-[1, 3, 2] diazaphosphorin [4, 5-b] quinoxaline 5,10-di-N-oxide -2,4(1H,3H)-dithione **7a** showed the best inhibitory effect out of all the compounds where VEGFR-2 activity was inhibited by 71% at 50 µM concentration, compared to control. Compound **5**also showed moderate inhibition of VEGFR-2 activity by 58% at 50 µM concentration, compared to control. The other compounds showed weak inhibition of VEGFR activity. Moreover, all the tested compounds displayed weak inhibition of Src activity.

Furthermore, IC50 determinations were done for the tested compounds being assayed by Src and VEGFR-2Kinases, the IC50 concentrations against Src were quite high, ranging from 143 µM to more than 3130 µM, Table **3**. On the other hand, compounds being assayed by VEGFR-2Kinase displayed IC50 values much lower than those with the Src kinase, ranging from 18 µM to more than 1580 µM. The best result observed was for the diazaphosphorin derivative **7a**, Table **3**. However, activity of this compound was significantly reduced upon substitution of the hydrogens of thiol and amino groups with methyl groups. Contrary to this observation the methylated derivative **8b** showed an inhibitory effect on Src kinase greater than its analogue **8a**.

2.3. Docking Studies

The vascular endothelial growth factor and its receptor tyrosine kinase VEGFR-2 or kinase insert domain receptor (KDR) are attractive targets for the development of novel anticancer agents [53]. The goal for researchers would be to find a selective inhibitor for the VEGFR-2 (KDR), because it is expressed on almost all endothelial cells and the majority of the effects in angiogenesis, including cell proliferation, micro-vascular permeability, invasion, migration, and survival are mediated by VEGFR-2 [54, 55]. In the present work, docking studies were performed to explore the binding mode between the 1,3,2-diazaphosphorine derivatives and KDR. The process was started by retrieving crystal structure of VEGFR-2 (PDB ID: 1VR2) from Protein Data Bank [56]. Docking studies of compounds with combination of VEGFR-2 (PDB ID: 1VR2) was performed using Molecular Operating Environment (MOE.10.2012) [57]. After

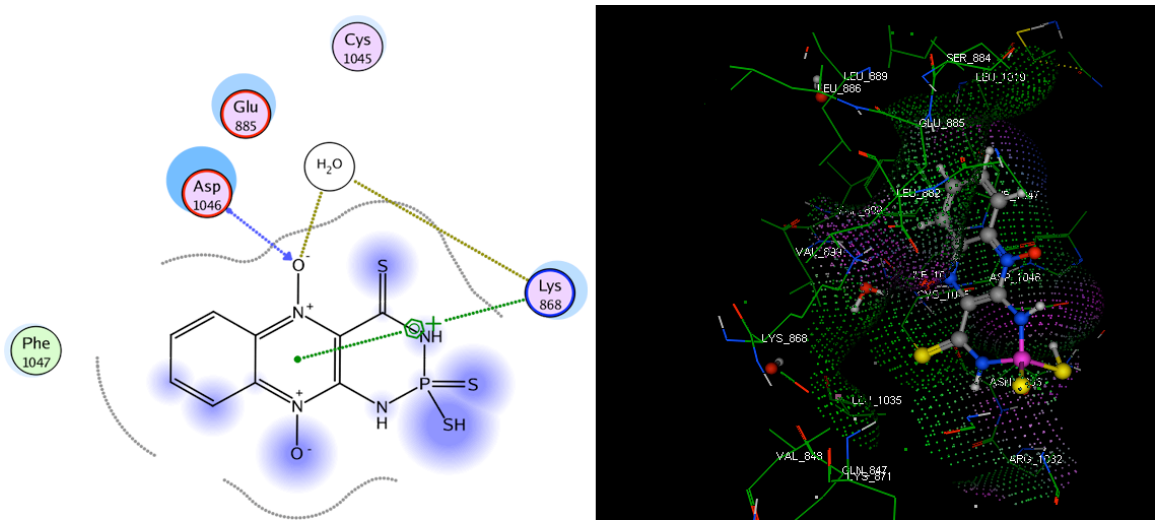


Fig. (3) (**a**) Two-dimensional representation of the interacting mode of **7a** with VEGFR-2 kinase. (**b**) Three-dimensional structural models of compound **7a** into VEGFR-2 kinase derived from the docking simulations.

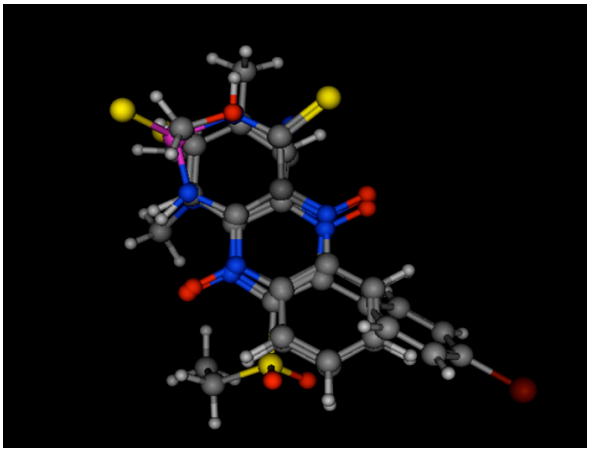


Fig. (4). Comparison of conformational alignments of compound **7a** with Q39 and TX-2098.

inspecting the kinase active site of several VEGFR-2 structures, 4 residues were selected: Lys868, Glu885, Cys919 and Asp1046, these residues interact consistently, by forming hydrogen bonds (H-bonds) with the inhibitors that are co-crystallized in the inspected VEGFR-2 structures [58, 59]. Thus, the compounds were docked in their most stable conformation into the ATP-binding site of VEGFR-2 and subjected to energy minimization. The best-fitted conformer (docking score -3.75 Kcal/mol) was observed for 2-mercapto- [1, 3, 2] diazaphosphorin [4, 5-b] quinoxaline-5, 10-di-N-oxide-2, 4 (1H,3H)-dithione **7a**. The docking interaction of compound **7a** with the active site of VEGFR-2 is represented in Fig. (**3**). When **7a** was bound to VEGFR-2, it formed hydrogen bonds with backbone-NH of Asp1046 (2.87 A°) and Lys 868 (2.30 A°), through a water molecule, as well as a strong ionic contact with Lys 868. The diazaphosphor seems to be extended toward the back hydrophobic pocket created by the conformational rearrangement of Phe1047 and Cys 1045 [60].

Moreover, comparison of conformational alignments of compound 7a and the quinoxalinederivatives Q39 and TX-2098 were also performed, Fig. (**4**).

3. EXPERIMENTAL

3.1. Chemistry

3.1.1. Chemical Methods

All the solvents used were commercially available and distilled before use. Reactions were monitored by thin-layer chromatography (TLC) on silica gel plates (60 F254), visualizing with ultraviolet light. Infra-red spectra (KBr) were recorded on FT-IR 5300 spectrophotometer and Perkin Elmer spectrum RXIFT-IR system (ν, cm^{-1}). ^{1}HNMR spectra were recorded on Varian Gemini spectrophotometer (300 MHz) in DMSO-d_6 as solvent. Proton chemical shifts (d) are relative to tetramethylsilane (TMS, d = 0.00) as internal standard and expressed in ppm. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet) as well as b (broad). Coupling constants (J) are given in hertz. Melting points were determined by using melting point apparatus and are uncorrected. MS spectra were obtained on a GC Ms-QP 1000 EX mass spectrometer at 70 eV. Microanalyses were performed using a C H N S/O analyzer. Elemental data are within ±0.4% of the theoretical values. All yields reported are unoptimized. The chemical reagents

used in synthesis were purchased from Fluka, Sigma and Aldrich**.**

3.1.2. Preparation of 3-amino-7-substituted-2-carbamoylquinoxaline 1, 4-dioxide (2a-c).

A mixture of 5(6)- substituted benzofuroxane**1a-c** and cyanoacetamide (10 mmol) was stirred for 10 min at 0°C. Over the cooled suspension was added a solution of triethylamine (5 drops) in dimethylformamide. The mixture was allowed to stand at room temperature over 24 h and then filtered off. The solid product was filtered and recrystallized from ethanol.

Compound 2a Yield 71%; mp. 260-265°C. IR (KBr, cm^{-1}):1660(C=O), 3388, 3224 (NH,NH_2).^{1}H NMR spectrum (DMSO-d6), δ, ppm 5.6 (s, 2H, NH_2, D_2O-exchangable), 8.53 (s, 2H, CO-NH_2, D2O-exchangable), 7.26-8.2 (m, 4H, quinox-H). Elemental Analysis: calcd, C, 49.09; H, 3.66; N, 25.45. $C_9H_8N_4O_3$ found C, 49.47; H,2.96; N, 25.40.

Compound 2b Yield 85%; mp. IR (KBr, cm^{-1}):1715(C=O), 1586, 1455 (NO), 3356, 3454, 3512 (NH,NH_2). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 5.76 (s, 2H, NH_2 D_2Oexchangable), 8.60 (s,2H, CO-NH_2, D_2O-exchangable), 7.36-8.32 (m, 3H, quinox-H). Elemental Analysis: calcd, C, 42.45; H, 2.77; N, 22.00. $C_9H_7ClN_4O_3$ found C 42.47; H 2.66; N, 22.12.

Compound 2c Yield 41%; mp 248°C. IR (KBr, cm^{-1}):1651(C=O), 3200, 3413 (NH,NH_2). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 3.93 (s, 1H, OCH_3), 4.9(s, 2H, NH_2-D_2O exchangable),7.85(s, 2H, CO-NH_2, D_2O-exchangable), 6.97-7.32 (m, 3H, quinox-H). Elemental Analysis: calcd, C, 48.00; H, 4.03; N, 22.39. $C_{10}H_{10}N_4O_4$ found C 48.27; H 4.46; N, 22.12.

3.1.3. Preparation of 7-chloro-2-mercapto[1,3,2]azathiaphosphorin[4,5-b]quinoxaline-5,10-di-N-oxide-4(1H)-one-2(3H)-thione, Pyridine solvate (3)

Dry pyridine (3 ml) was added slowly and carefully with stirring to carefully ground and stirred mixture of 2-amino-3-carbamoyl-6-chloro quinoxaline 1, 4-dioxide **2b** (2.18g, 0.01 mol) and phosphorus pentasulfide (3.33 g,0.015 mol). The mixture was heated and changed to oil. After addition of pyridine, the mixture was boiled for 5 min and then washed with boiling benzene (2 × 100 ml); water (200 ml) was added and this was boiled for 2 min and then cooled down. The precipitate was filtered off and carefully washed with water and ethanol. Yield (83%); mp 360°C (DMF–water, 3:1). IR (KBr, cm^{-1}): 1655 (CO), 1625(C=N), 1500, 1358 (NO), 3408(NH), 1244 (C=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 7.39(1H, d, H_9), 7.50(1H, t, γ-H), 7.58 (1H, s, H_8), 7.73(1H, t, β-H), 7.95(2H, d, α-H), 7.99(1H, d, H_6), 10.18 (1H, br. s, NH, D_2O-exchangable). MS (m/z): 365 (M^+), 204 (M^+ - SH,- O_2 –PS_2), 195 (M^+ -O_2, -NH,-CO,-PS_2), 188 (M^+ -SH, -O_3,- PS_2), 63 (PS^+). Elemental Analysis: calcd, C, 37.80; H, 2.27; N, 12.59; S, 21.62. $C_{14}H_{10}ClN_4O_3PS_3$ found C, 37.78; H, 2.31; N, 12.65; S, 21.73

3.1.4. Preparation of 3-amino-7-substituted-2-quinoxalinecarbonitrile -1, 4-di-N-oxides (4a-c) [48].

A mixture of 5(6)-substituted benzofuroxane**1a-c** (10 mmol) and malononitrile (10.6 mmol) was stirred for 10 min at 0°C. Over the cooled suspension was added a solution of triethylamine (5 drops) in dimethylformamide. The mixture was allowed to stand at room temperature over 24 h and then filtered off. The solid product was washed with diethylether and recrystallized from dioxane.

Compound 4a Yield 65%; m.p. 190°C.

Compound 4b Yield 80%; m.p. 263-264°C.

Compound 4c Yield 31%; m.p. 248-249°C.

3.1.5. Preparation of 7-chloro-2-mercapto[1,3,2]diazaphosphorin[4,5-d]quinoxaline-5,10-di-N-oxide-2,4(1H, 3H)-dithione (5)

A mixture of 2-amino-3-carbamoyl-6-chloro quinoxaline 1,4-dioxide **2b** (2.18g, 0.01 mol) and phosphorus pentasulfide (3.33g,0.015 mol) in dimethylformamide were carefully ground and stirred. The mixture was boiled for 5 min and then washed with boiling benzene (2 × 100 ml); water (200 ml) was added and this was boiled for 2 min and then cooled down. The precipitate was filtered off and carefully washed with water and ethanol. Yield (34%); mp160°C. IR (KBr, cm^{-1}): 1655 (CO), 1625(C=N), 1500, 1490 (NO), 3408(NH), 1244(C=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 7.57(1H, d, H_8), 7.70(1H,s, H_6), 7.96 (1H, d, H_9), 9.18 (1H, br. s, NH, D_2O-exchangable). MS (m/z): 365 (M^+), 204 (M^+ - SH,- O_2 –PS_2), 195 (M^+ -O_2, -NH,-CO,- PS_2), 188 (M^+ -SH, -O_3,- PS_2), 63 (PS^+). Elemental Analysis: calcd, C, 37.80; H, 2.27; N, 12.59; S, 21.62. $C_{14}H_{10}ClN_4O_3PS_3$ found C, 37.78; H, 2.31; N, 12.65; S, 21.73.

3.1.6. Preparation of 2-amino-3-carbamothioyl-6-chloroquinoxaline 1,4-dioxide (6)

The same procedure for preparation of **5**, the major product obtained **6**; Yield (66%); mp 250°C. (ethanol). IR (KBr, cm^{-1}): 3125, 3422 (NH_2), 1254(C=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 7.48 (1H, d, H_8), 7.70 (1H,s, H_6), 7.96 (1H, d, H_9), 5.42 (1H, br. s, NH_2, D2O exchangeable). MS (m/z): **270** (M^+), 255 (M^+ - NH_2), 163 (M^+ -O_2, -NH_2,-CS). Elemental Analysis: calcd, C, 39.93; H, 2.61; N, 20.70; S, 11.85. found C, 39.89 ; H, 2.59; N, 20.73; S, 11.79

3.1.7. Preparation of 2-mercapto-7-substituted [1, 3, 2] diazaphosphorin [4,5-b] quinoxaline 5, 10-di-N-oxide - 2,4(1H,3H)-dithione, Pyridine solvate (7a-c)

Dry pyridine (3 ml) was added slowly and carefully with stirring to carefully ground and stirred mixture of 3-amino-7-substituted-2-quinoxalinecarbonitrile-1, 4-di-N-oxides **4a-c** (10 mmol) and phosphorus pentasulfide (15 mmol). The mixture was heated and changed to oil. After addition of pyridine, the mixture was boiled for 5 min and then washed with boiling benzene (2 × 100 ml); water (200 ml) was added and this was boiled for 2 min and then cooled down. The precipitate was filtered off and carefully washed with water and ethanol. **Compound 7a** Yield (63%); mp 160°C (DMF–water, 3:1). IR (KBr, cm^{-1}): 1635 (C=N), 3364, 3317, 3231 (2 NH), 1544, 1484 (NO), 1228(C=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 10.0 (1H, br. s, NH-P, D_2O exchangable), 10.18 (1H, br. s, NH, D2O-exchangable), 7.39-7.83 (7H, m, Ar-H and pyridine-H), 7.8(2H,d, H_6, H_9). MS (m/z): 330 (M^+), 265 (M^+ - SH, -O_2), 205 (M^+ -SH,-O_2, -CSNH2), 170 (M^+ - SH,- O_2 –PS2), 144 (M^+ -O_2, CS–NH–

PS2), 95 ($PS2^+$). Found, %: C; H; N; S.C 39.47; H 2.66; N, 13.12; S 30.01. $C_{14}H_{11}N_4O_2PS_3$. Calc., %: C 39.43; H 2.60; N 13.14; S 30.07.

Compound 7b. Yield (77%); mp 210°C (DMF–water, 3:1).IR (KBr, cm^{-1}): 1615(C=N), 1538, 1496 (NO), 3124,3249,3385(2 NH), 1226 (C=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 7.41-7.84(6H, m, H_8,H_9 and pyridine-H), 7.85 (1H, s, H_6), 8.59(2H, d, α-H), 10.11 (1H, br. s, NH-P, D2O-exchangable), 10.43(1H, br. s, NH, D2O-exchangable). MS (m/z): 364 (M^+), 299 (M^+ -SH, -O_2), 238 (M^+ -SH,-O_2, -CSNH2), 204 (M^+ - SH, -O_2 –PS2), 95 ($PS2^+$). Elemental Analysis: calcd C, 36.48; H, 2.19; N, 12.15; S, 27.83, $C_9H_6ClN_4O_2PS_3$ found C, 36.52; H, 2.18; N, 12.33; S, 27.91

Compound 7c Yield (23%); mp 130°C (DMF–water, 3:1). IR (KBr, cm^{-1}): 1623(C=N), 1557, 1490(NO), 3350,3221(2 NH), 1251 (C=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 3.9(3H, s, OC<u>H</u>$_3$), 7.39(1H, d, H_8), 7.59(1H, d, H_9), 7.79 (2H, t, β-H), 7.8(1H, s, H_6), 8.06(1H, t, ɣ-H), 8.59(2H, d, α-H), 10.11 (1H, br. s, NH-P, D2O-exchangeable), 10.43(1H, br. s, NH, D2O exchangeable). MS (m/z): 364 (M^+), 299 (M^+ -SH, -O_2), 238 (M^+ -SH,-O_2, -CSNH2), 204 (M^+ - SH,- O_2 –PS2), 95 ($PS2^+$). Elemental Analysis: calcd, C, 39.46; H, 2.87; N, 12.27; S, 28.10. $C_9H_6ClN_4O_2PS_3$ found C, 39.48; H, 2.90; N, 12.32; S, 27.81.

3.1.8. Preparation of 2, 4-dimethylthio-5-methyl-7-substituted [1, 3, 2] diazaphosphorin [4, 5-b]quinoxaline 5,10-di-N-oxide-2- thione (8a,b)

Pyridine solvate **7a, b** (0.01mol) was dissolved in aqueous (1 mol/l) solution of NaOH (80 ml). The solution obtained was washed twice with benzene and cooled down to 0-5°C, and then dimethyl sulfate (5 ml) was added. The reaction mixture was stirred for 30 min, the precipitate was filtered off and washed with water and ethanol.

Compound 8a Yield (54%); mp 240-242°C(DMF–water, 3:1).IR (KBr, cm^{-1}): 1643(C=N), 1243 (P=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 2.07 (3H, d, P–SC<u>H3</u>); 2.49 (3H, s, 4-C–SC<u>H3</u>); 3.22 (3H, d, N^1–CH3), 7.39(1H, t, H_8), 7.42(1H, d, H_6), 7.63(1H, t, H_7), 7.85(1H, d, H_9), Elemental Analysis: Found, %: C 37.65; H 4.94; N 17.46; S 30.12. C10H15N4PS3. Calculated, %: C 37.72; H 4.75; N 17.60; S 30.21.

Compound 8b Yield (43%); mp 262-265°C (DMF–water, 3:1). IR (KBr, cm^{-1}): 1633(C=N), 1444, 1371 (NO), 1243 (P=S). ^{1}H NMR spectrum (DMSO-d6), δ, ppm 2.08 (3H, s, P–SC<u>H3</u>); 2.51 (3H, s, 4-C–SC<u>H3</u>); 3.28 (3H, s, N^1–CH3), 7.39(1H, d, H_9), 7.68(1H, d, H_8), 7.73(1H, s, H_6), Elemental Analysis: Found, %: C 37.65; H 4.94; N 17.46; S 30.12. C10H15N4PS3. Calculated, %: C 37.72; H 4.75; N 17.60; S 30.21.

3.2. Biological Studies

3.2.1. Cytotoxicity against PC-3 Prostate Adenocarcinoma Cell Line

The cytotoxic inhibitory effect of the new synthesized compounds on human prostate cancer PC-3 was performed at the pharmacology department, Ain Shams University, Cairo, Egypt.

Materials:

RPMI-1640 medium, fetal bovine serum (FBS) and phosphate buffered saline were purchased from Lonza (Basel, Switzerland). Dimethyl sulfoxide and SulphoRhodamine-B (SRB) were obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY, USA).

Cell culture:

PC3, human prostate cancer cell line was grown in RPMI-1640 medium supplemented with 10% heat inactivated FBS, 100 units/mL of penicillin and 100 mg/mL of streptomycin and maintained at 37° in a humidified atmosphere containing 5% CO2. The cells were maintained as "monolayer culture" by serial subculturing.

SRB cytotoxicity assay:

Cytotoxicity was determined using SRB method as previously described by Skehan *et al.* (1). Exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates at 1000-2000 cells/well in RPMI-1640 supplemented medium. After 24 h, cells were incubated for 72 h with various concentrations of the tested compounds. Following 72 h treatment, the cells will be fixed with 10% trichloroacetic acid for 1 h at 4 ºC. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris-HCl for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA).

Data Analysis

The dose response curve of compounds was analyzed using Emax model. Where R is the residual unaffected fraction (the resistance fraction), [D] is the drug concentration used, Kd is the drug concentration that produces a 50% reduction of the maximum inhibition rate and m is a Hill-type coefficient. IC50 was defined as the drug concentration required to reduce fluorescence to 50% of that of the control (i.e., Kd = IC50 when R=0 and Emax =100-R) (2).

$$\%\ \text{Cell viability} = (100 - R) \times \left(1 - \frac{[D]^m}{K_d{}^m + [D]^m}\right) + R$$

3.2.2. Profiling Against the Protein Kinases VEGFR and Src

The evaluation of the new synthesized compounds were perfomed at Kinexus compound profiling services and evaluation of compounds against protein kinase targets, order no° 2618.

Kinase Activity Assay Protocol

- **Materials**

Quality Control & Reagents

The various protein kinase targets to be employed in the compound profiling process were cloned, expressed and purified using proprietary methods. Quality control testing is

routinely performed on each of the targets to ensure compliance to acceptable standards. Protein substrates employed in the compound profiling process were synthesized internally, KDR, active (aliases include FLK1 and VEGFR) recombinant human protein expressed in Sf9 cells and Src, active (aliases include ASV, SRC1, c-SRC, and p60-Src) full length recombinant protein expressed in E.coli cells. 33P-ATP was purchased from PerkinElmer. All other materials were of standard laboratory grade. The highly purified active enzymes are generated from the full-length human genes and are mutation free.

- **Methods**

The assay condition for the various protein kinase targets were optimized to yield acceptable enzymatic activity. In addition, the assays were optimized to give high signal-to-noise ratio.

- **Protein Kinase Assays**

A radioisotope assay format was used for profiling evaluation of protein kinase targets and all assays are performed in a designated radioactive working area. Blank control was set up for each protein kinase target which included all the assay components except the addition of the appropriate substrate (replaced with equal volume of assay dilution buffer). The corrected activity for each protein kinase target was determined by removing the blank control value.

3.3. Molecular Modeling

All the molecular modeling calculations and docking simulation studies were performed using Molecular Operating Environment (MOE®) [57] version 10.2010, Chemical Computing Group Inc., Montreal, Canada. All the interaction energies and different calculations were automatically calculated.

The target compounds were constructed into a 3D model using the builder interface of the MOE program, the target compounds were subjected to a conformational search and all conformers were subjected to energy minimization, all the minimizations were performed. The X-ray crystal-lographic structures of VEGFR-2 receptor (PDB ID: 1VR2) was obtained from the Protein Data Bank.

CONCLUSIONS

Recently, the concept of angiogenesis was introduced as a pathway in cancer treatment. New derivatives of 1, 3, 2-diazaphosphorin [4, 5-b] quinoxaline-5, 10-di-N-oxide were synthesized and evaluated for the treatment of prostate cancer through inhibition of the tyrosine kinases VEGFR-2 and SRC, which are involved in angiogenesis and metastases of certain tumors. The results of cytotoxicity against human prostate cell line PC-3 showed that, compounds **7a**, **8a** and **5** were the most potent cytotoxins. In particular, 2-mercapto-1, 3, 2-diazaphosphorin [4, 5-b] quinoxaline-5,10-di-N-oxide-2,4-dithione **7a** demonstrated good inhibitory effect on VEGFR-2. However, the activity of this series of compounds did not seem to be mediated through SRC kinase inhibition.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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