

Bull. Chem. Soc. Ethiop. 2018, 32(2), 285-308.
© 2018 Chemical Society of Ethiopia and The Authors
DOI: <https://dx.doi.org/10.4314/bcse.v32i2.9>

ISSN 1011-3924
Printed in Ethiopia

DISCOVERY OF NEW THIOPHENE, PYRAZOLE, ISOXAZOLE DERIVATIVES AS ANTITUMOR, c-Met, TYROSINE KINASE AND Pim-1 KINASE INHIBITORS

Rafat Milad Mohareb^{1*}, Khalid M. H. Hilmy² and Yasser Abdelaziz Elshehawy¹

¹Department of Chemistry, Faculty of Science, Cairo University, Giza, Egypt

²Department of Chemistry, Faculty of Science, Menufiya University, Shebin El-Kom, Egypt

(Received February 8, 2018; Revised March 18, 2018; Accepted March 22, 2018)

ABSTRACT. The reaction of cyclohexan-1,3-dione (1) with ethyl orthoformate (2) in acetic acid gave the 2-(ethoxymethylene)cyclohexane-1,3-dione (3). The latter compound was used for further heterocyclization reactions to give thiophene, pyrazole and pyran derivatives. The cytotoxicity of the newly synthesized compound against the six cancer cell lines NUGC, DLDI, HA22T, HEPG2, HONE1 and MCF showed that compounds **5**, **10c**, **10d**, **13b**, **14a**, **18b**, **18d**, **18e** and **20b** were the most potent compounds. On the other hand, the toxicity of these compounds against shrimp larvae indicated that compounds **7a**, **10c**, **13b**, **14a**, **18b** and **18d** were non toxic against the tested organisms. Inhibition of the most potent compounds towards the tyrosine kinases c-kit, FIT-3, Vascular Endothelial Growth Factor Receptor (VEGFR)-2, Estimated Glomerular Filtration Rate (EGFR) and Platelet-Derived Growth Factor Receptor (PDGFR) revealed that compounds **5**, **10c**, **10d**, **13b**, **18b**, **18d**, **18e** and **20b** were of the highest inhibitory effect. The Pim-1 kinase test revealed that compounds **10d**, **18b** and **20b** were of the highest inhibitory effect. In addition, the c-Met enzymatic activities showed that compounds **10c**, **10d**, **18b**, **18e**, **19** and **20b** showed higher potencies against c-Met kinase than the reference foretinib. On the other hand, compounds **7a**, **7b**, **10d**, **13a**, **13b**, **14a**, **14b**, **16a**, **16b**, **17**, **18a-f**, **19** and **20** showed higher inhibition towards PC-3 cell line than the reference SGI-1776. Compounds **10c** and **18b** were of common potencies and their molecular docking was described.

KEY WORDS: Cyclohexane 1,3-dione, Pyrazole, Thiophene, Cytotoxicity, Tyrosine kinases

INTRODUCTION

Major progress in cancer chemotherapy requires new drugs to eradicate the entire neoplastic diseases in human being. Finding a novel structure leads that may be of use in designing new, potent, selective, and less toxic anticancer agents remains a major challenge for medicinal chemistry researchers [1-4]. Random screening of natural products and synthetics has been the source of new leads in approaches to drug discovery [5-7]. In our ongoing search for the new potential antitumor agents, we therefore aimed to develop a series of new synthetic leads which are more accessible and more amenable to optimization through analog synthesis.

Many planar heteroaromatic derivatives have shown anti-proliferative activity *in vitro* and some of them are important anticancer drugs [8-10]. Some of these types of compounds have the indazole nucleus which is a structural component of a large number of biologically active natural and unnatural compounds. Recently the high anti-proliferative activity of nitrogen containing heterocycles in two human breast cancer cell lines (MDAMB 231 and MCF-7) was reported and the authors were able to show that the tubulin is the primary target of these agents inhibiting its polymerization [11-13]. In the previous state of the art literature, the heterocycles containing sulfur and nitrogen possess a broad range of pharmaceutical activities. The family of sulfur heterocycles includes highly stable aromatic compounds that display physicochemical properties with relevance in the design of new materials, especially those relating to cancer treatments [14]. Among the sulfur heterocycles, benzo[*b*]thiophene is a unique heterocyclic core that has been pictured as an important pharmacophore of some bioactive molecules and therefore fascinated much interest [15, 16]. Thiophene containing compounds are well known to

*Corresponding author. E-mail: raafat_mohareb@yahoo.com

This work is licensed under the Creative Commons Attribution 4.0 International License

exhibit various biological activities such as BACE1 inhibitors [17] and anti-breast cancer [18]. Conversely, pyrazole derivatives have been reported in the literature to demonstrate anti-tumor [19] and kinase inhibitions [20]. Motivated by these results and in continuation of our previous work aimed at the synthesis of a new heterocyclic systems for anti-tumor evaluations [21-23] we report here the modification of cyclohexane-1,3-dione into a variety of novel condensed pyrazole, thiophene and isoxazole derivatives incorporating the cyclohexane moiety. It is relied on combination of different pharmacophores of bioactive compounds [24-27] to obtain new compounds with potent activity, increased selectivity and reduced adverse effects. Following this strategy and in a continuation of our previous study, we synthesized heterocyclic derivatives in most of the work using hydrazine and/or cyano derivatives [28-30] with cyclohexane-1,3-dione aiming to synthesis heterocyclic scaffolds in order cumulate all the expected activities of the individual rings onto one structure. The anti-tumor evaluations followed by tyrosine kinase, Pim-1 kinases and c-Met inhibitions were measured for the newly synthesized target molecules. Additionally the confirmation of the structure of the newly synthesized compounds using different spectroscopic tools was described.

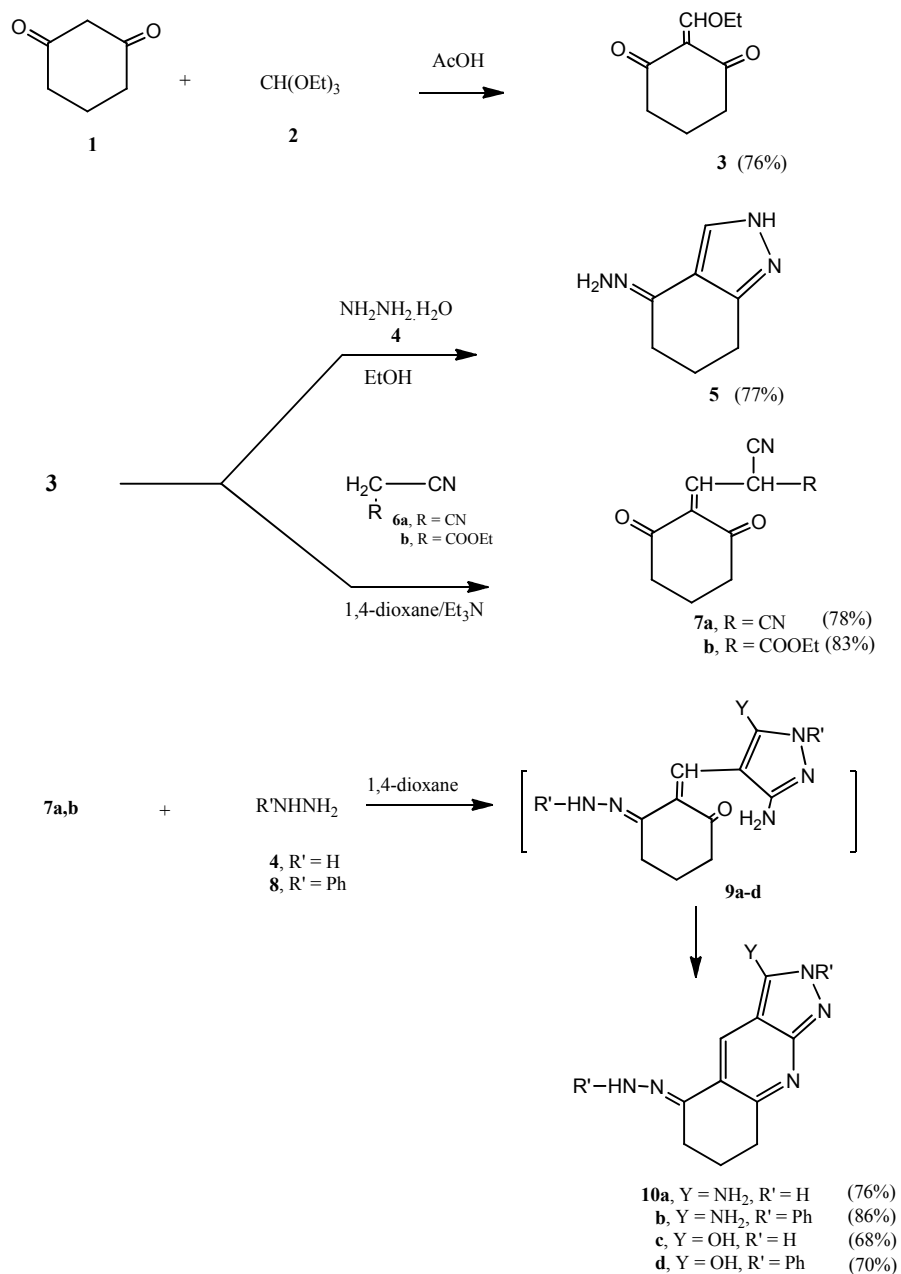
RESULTS AND DISCUSSION

In the present work, we are presenting the uses of cyclohexane-1,3-dione for the synthesis of pyrazole, thiophene, isoxazole and pyran derivatives using simple reaction sequences followed by the evaluation of the newly synthesized products against cancer cell lines and some kinases inhibitions. Thus, the reaction of cyclohexane-1,3-dione (**1**) with ethyl orthoformate (**2**) in acetic acid gave the 2-(ethoxymethylene)cyclohexane-1,3-dione (**3**) [31]. The structure of the latter product was based on its analytical and spectral data. The ^1H NMR spectrum revealed, beside the expected signals, a triplet at δ 1.28 ppm indicating the presence of the CH_3 , a quartet at δ 3.89 ppm for the CH_2 group and a singlet at δ 6.79 ppm due to the presence of the CH group. Moreover, the ^{13}C NMR spectrum showed the presence of δ at 16.3 due to the presence of the OCH_2CH_3 group, δ 62.8 indicating the presence of the OCH_2CH_3 group, two signals at δ 112.3, 158.2 due to the $\text{C}=\text{CH}$ group and two signals at δ 177.3, 179.2 indicating the presence of the two CO groups. Compound **3** with the two active centers, the $\text{C}=\text{O}$ group and the ethoxymethino moiety showed an interesting reactivity toward hydrazine hydrate. Thus, compound **3** reacted with hydrazine hydrate to give the 4-hydrazono-4,5,6,7-tetrahydro-2*H*-indazole (**5**) which was identified on the basis of its analytical and spectral data (see Experimental). The reaction of compound **3** with either of malononitrile (**6a**) or ethyl cyanoacetate (**6b**) gave the alkylated products **7a** and **7b**, respectively. Compounds **7a** and **7b** with different active sites towards nucleophilic reagents encourage us for their reactions with two folds of either of hydrazine hydrate (**4**) or phenylhydrazine (**8**) affording the 5-hydrazono-5,6,7,8-tetrahydro-2-substituted-pyrazolo[3,4-*b*]quinoline derivatives **10a-d**, respectively (Scheme 1). Formation of **10a-d** occurred via the intermediacy of **9a-d** followed by cyclization. The structures of **10a-d** were established on the basis of their respective analytical and spectral data (see Experimental). Moreover, the reaction of either of compound **7a** or **7b** with hydroxylamine hydrochloride (**11**) gave the isoxazolo[3,4-*b*]quinolin-5(6*H*)-one oxime derivatives **13a** and **13b**, respectively through the intermediacy of **12a,b**. The analytical and spectral data were consistent with their respective structures.

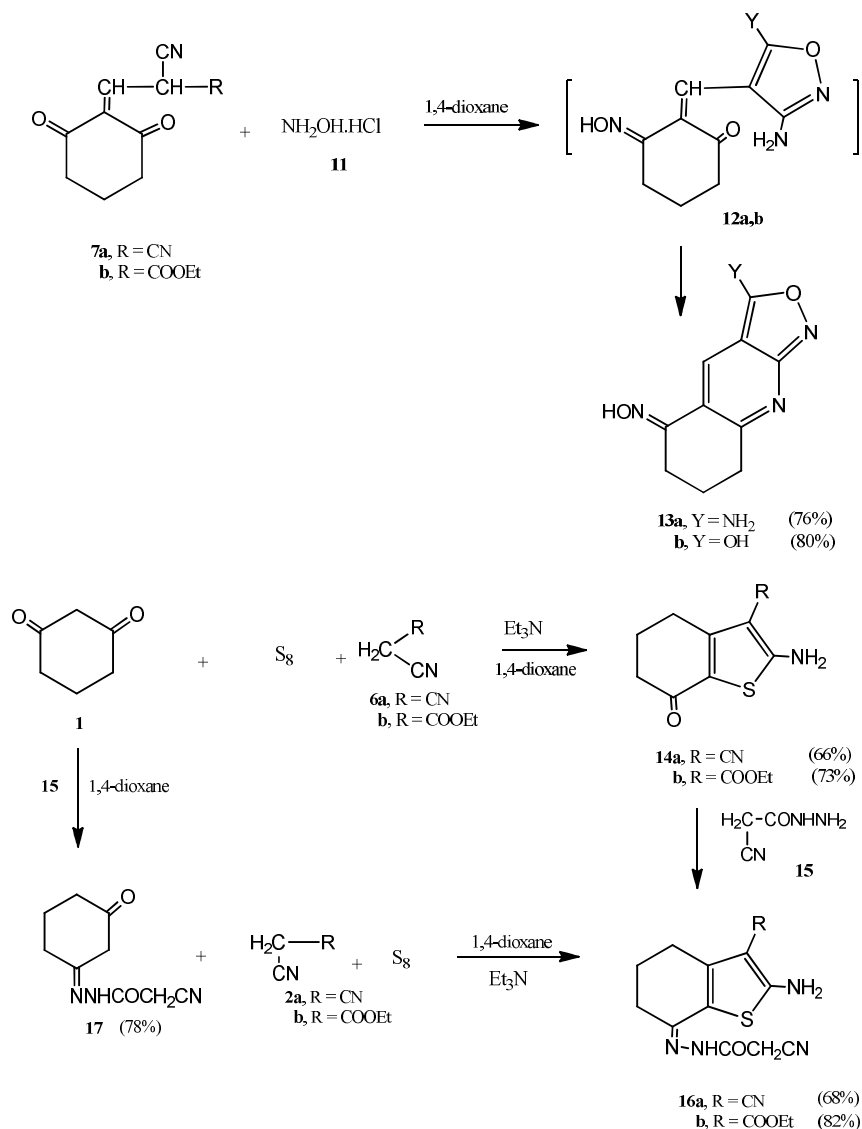
Next, we studied the Gewald's thiophene [32-34] synthesis using cyclohexane-1,3-dione (**1**). Thus, the reaction of compound **1** with either of malononitrile (**6a**) or ethyl cyanoacetate (**6b**) gave the thiophene derivatives **14a** and **14b**, respectively.

The reaction of either of compound **14a** or **14b** with cyanoacetylhydrazine (**15**) gave the hydrazide-hydrazone derivatives **16a** and **16b**, respectively (Scheme 2). The structures of compounds **16a** and **16b** were based on their respective analytical and spectral data. Thus, the ^1H NMR spectrum of **16a** revealed the presence of a singlet at δ 4.42 ppm for the CH_2 group of

the cyanoacetamido moiety, a singlet at δ 8.31 ppm (D_2O exchangeable) indicating the presence of the NH group. In addition the ^{13}C NMR spectrum which showed a signal at δ 58.8 equivalent

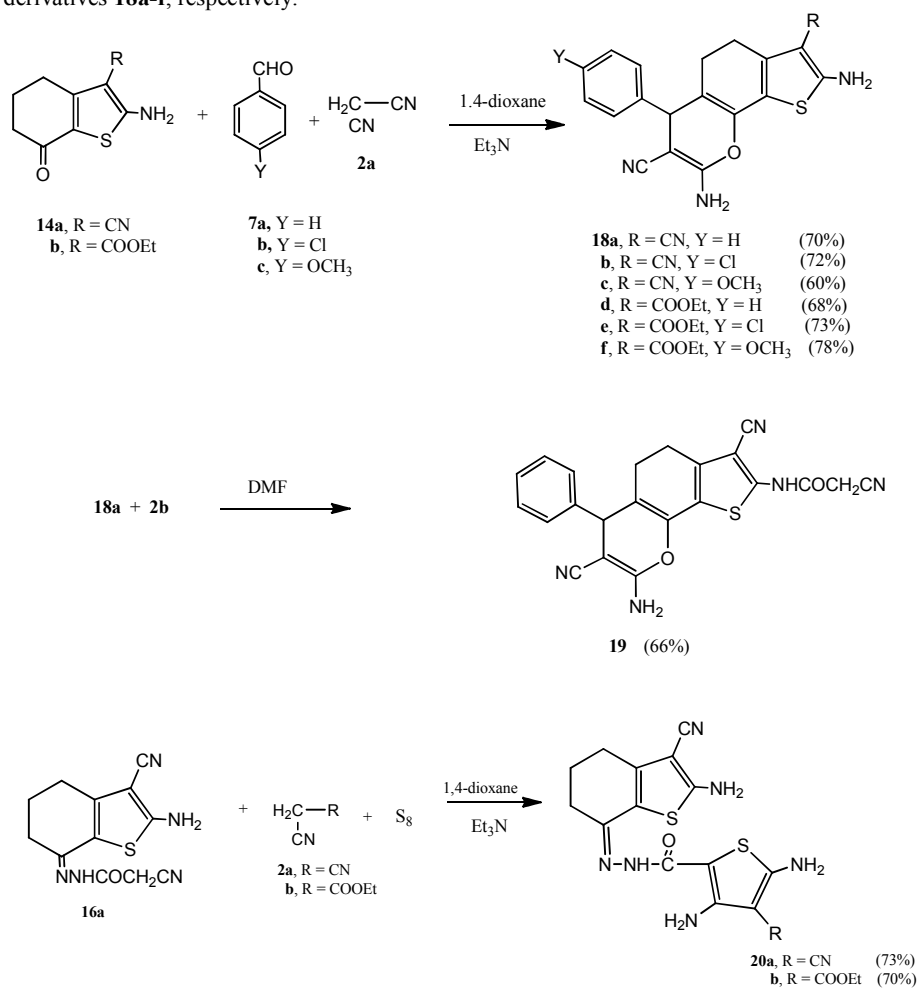


Scheme 1. Synthesis of compounds **3**; **5**; **7a,b** and **10a-d**.

Scheme 2. Synthesis of compounds **13a,b**; **14a,b**; **16a,d** and **17**.

to the cyanoacetamido CH₂ group, the presence of the two CN groups at δ 116.6, 117.4 and two signals at δ 164.8, 172.3 equivalent to the C=O and C=N groups, respectively. Further elucidation for the structures of compounds **16a** and **16b** was obtained through their synthesis via another reaction route. Thus, the reaction of molar ratio cyclohexane-1,3-dione (**1**) and cyanoacetylhydrazine (**15**) gave the hydrazide-hydrazone derivative **17**. The latter compound reacted with either of malononitrile (**2a**) or ethyl cyanoacetate (**2b**) and elemental sulfur to give the same thiophene derivatives **16a** and **16b**, respectively (m.p., mixed m.p. and finger print IR spectra).

It is well known that multi-component reactions (MCRs) can combine three or more components together in a single reaction vessel and produce final products with a minimum of synthetic time and effort [35] because they are no need to separate any reaction intermediate [36]. Such MCRs often result in high atom economy and high selectivity products [37]. They are also applicable to the synthesis of heterocyclic systems [38]. It is quite remarkable that many top-selling pharmaceuticals contains 4-*H* pyran derivatives [39-43] this encouraged us to synthesis 4-*H* pyran derivatives through the multi-component reactions of compounds **14a,b**. Therefore, the reaction of either of compound **14a** or **14b** with any of the aromatic aldehydes **7a**, **7b** or **7c** and malononitrile (**2a**) gave the 5,6-dihydro-4*H*-thieno[3,2-*h*]chromen-8-amine derivatives **18a-f**, respectively.



Scheme 3. Synthesis of compounds **19a-f**, **19** and **20a,b**.

The two amino groups in compounds **18a-f** were of different nucleophilicity. The amino group located at C-2 close to the aromatic thiophene moiety showed more nucleophilicity than the amino group at C-8 close the pyran moiety. It is noteworthy that the amino group in the 8-

aminopyran moiety possesses much lower nucleophilicity compared to aliphatic amines due to its lower location at the C-2 carbon bonded to the oxygen [44] such amino group usually requires a catalytic base to initiate its nucleophilicity [45]. Thus, throughout our work, the reaction of compound **18a**, as a derivative of **18a-f**, with ethyl cyanoacetate (**2a**) without the use any catalyst in dimethylformamide under reflux gave the 2-cyanoacetamido derivative **19**.

Compound **16a** was capable for further thiophene synthesis, due the presence of the cyanomethylene moiety, through its reaction with either of malononitrile (**2a**) or ethyl cyanoacetate (**2b**) and elemental sulfur to give the 5-amino-3-cyanothiophene-2-carboxamide derivatives **20a** and **20b**, respectively (Scheme 3).

In vitro cytotoxic assay

Table 1. Cytotoxicity of novel compounds against a variety of cancer cell lines^a [IC₅₀^b (nM)].

Compound	Cytotoxicity (IC ₅₀ in nM)						
	NUGC	DLDI	HA22T	HEPG2	HONE1	MCF	WI38
3	2160	3210	2765	1541	1220	2659	na
5	42	68	584	228	167	268	na
7a	420	428	325	118	529	168	na
7b	2138	2279	1340	250	1549	1618	na
10a	1242	2360	2157	3212	1287	2130	na
10b	1107	2150	2148	2138	1268	2190	na
10c	124	121	38	155	178	420	na
10d	120	58	210	320	79	154	na
13a	1280	2360	3268	3385	2428	2530	na
13b	66	222	1124	217	2254	2128	na
14a	88	650	463	160	318	130	na
14b	1870	990	2158	1380	2442	1639	na
16a	1150	408	1319	2170	2048	1149	868
16b	1035	3160	2088	2116	2180	1274	na
17	1238	2254	2237	2429	1179	2480	na
18a	1335	3280	2016	1380	4270	3240	na
18b	314	155	128	70	69	154	na
18c	1128	273	168	528	318	80	na
18d	84	1490	2168	1299	2034	2868	na
18e	118	219	364	306	260	1055	na
18f	1282	1290	2369	1046	1077	2362	na
19	1028	2428	2118	1090	1428	2248	na
20a	1072	1169	2529	1165	550	280	na
20b	128	270	56	1068	148	440	na
Foretinib	23	258	48	240	35	63	na

^aNUGC, gastric cancer; DLDI, colon cancer; HA22T, liver cancer; HEPG2, liver cancer; HONE1, nasopharyngeal carcinoma; HR, gastric cancer; MCF, breast cancer; WI38, normal fibroblast cells. ^bThe sample concentration produces a 50% reduction in cell growth. na = not active.

Structure activity relationship

It is clear from Table 1 that the cytotoxicity of the newly synthesized products against NUGC, DLDI, HA22T, HEPG2, HONE1 and MCF using foretinib as the reference showed that compounds **5**, **7a**, **10c**, **10d**, **13b**, **14a**, **18b**, **18d**, **18e** and **20b** were the most cytotoxic compounds among the tested compounds. The 2-(ethoxymethylene)cyclohexane-1,3-dione (**3**) showed very low cytotoxicity towards the six cancer cell lines, however, its reaction with hydrazine hydrate to give the 4-hydrazono-4,5,6,7-tetrahydro-2*H*-indazole derivative **5** which is the nitrogen rich compound with high cytotoxicity towards the six cancer cell lines. It is of great

value to note that compound **5** showed higher cytotoxicity against HEPG2 cell line than foretinib with IC_{50} 228 nM. It is clear that the fused pyrazole derivative **5** showed higher cytotoxicity than the 2-(ethoxymethylene)cyclohexane-1,3-dione (**3**). On the other hand, the reaction of compound **3** with either of malononitrile or ethyl cyanoacetate gave compounds **7a** and **7b** with different potencies. Thus, it is obvious that compound **7a** with the dicyano moieties showed remarkable cytotoxicity higher than **7b** towards the six cancer cell lines, although compound **7b** showed high potency against HEPG2 cell line with IC_{50} 250 nM by a value that very close to that of foretinib. Considering, nitrogen rich compounds, the 5,6,7,8-tetrahydro-pyrazolo[3,4-*b*]quinoline derivatives **10a-d**, it is clear that compounds **10c** ($Y = OH$, $R' = H$) and **10d** ($Y = OH$, $R' = Ph$) showed the highest cytotoxicity among the four compounds. The high potencies of compounds **10c** and **10d** were attributed to the presence of the OH group in these compounds. Compound **10d** showed a higher cytotoxicity than foretinib towards DLDI with IC_{50} 58 nM. In general compound **7a** showed the highest cytotoxicity which is close that those of the tricyclic compounds **10c** and **10d**. Reaction of either of compound **7a** or **7b** with hydroxylamine hydrochloride gave 5,6,7,8-tetrahydroisoxazolo[3,4-*b*]quinoline derivatives **13a** and **13b** where compound **13b** ($Y = OH$) showed higher cytotoxicity than **13a** towards the three cancer cell lines NUGC, DLDI, HEPG2 with IC_{50} 's 66, 222, and 217 nM, respectively. It is obvious that tricyclic pyridinopyrazole derivatives **10c,d** showed higher cytotoxicity toward the six cancer cell lines than the the tricyclic pyridinoisoxazole derivative **13b** as the latter showed low potency against HONE1 and MCF cell lines.

Considering the thiophene derivatives **14a,b** it is clear that compound **14a** ($R = CN$) showed higher cytotoxicity against the six cancer cell lines than compound **14b**. The reactivity of **14a** was attributed to the presence of the electronegative CN group. For the hydrazide-hydrazone derivatives **16a** and **16b** it is obvious that compound **16a** ($R = CN$) showed high cytotoxicity only towards DLDI cell line with IC_{50} 408 nM while compound **16b** ($R = COOEt$) showed low potencies against the six cancer cell lines. On the other hand, both of the hydrazidehydrazone derivative **17** showed low potencies towards the six cancer cell lines. Comparison of the thiophene derivatives **14a,b**, the hydrazide-hydrazone derivatives **16a,b** and **17** revealed that the thiophene derivative **14a** with the highest cytotoxicity among the five compounds.

Considering the multi-component products the thieno[3,2-*h*]chromene-3-carbonitrile derivatives **18a-f**, it is clear that compounds **18b** ($R = CN$, $Y = Cl$) and **18c** ($R = CN$, $Y = OCH_3$) showed the highest cytotoxicity among the six compounds. On the other hand, compound **18d** ($R = COOEt$, $Y = H$) showed high cytotoxicity against NUGC cell line with IC_{50} 84 nM but compound **18e** ($R = COOEt$, $Y = Cl$) revealed high cytotoxicity against the five cancer cell lines NUGC, DLDI, HA22T, HEPG2 and HONE1. It is obvious through compounds **18a-f** that the presence of either the CN or Cl groups enhances the high potencies of compounds **18b** and **18c**. The thieno[3,2-*h*]chromen-8-yl)-2-cyanoacetamide derivative **19** exhibited low potency towards the six cancer cell lines. It is clear that the cyanoacetamido moiety reduce the potency of compound **19**. It is obvious that compounds **18b,d,e** showed the highest cytotoxicity among the pyran derivatives **18a-d** and **19**. Considering the dihydrobenzo[*b*]thiophen-7(4*H*)-ylidene)thiophene-2-carbohydrazide derivatives **20a,b** with the two thieno moieties showed activities towards some selective cell lines. Thus, compound **20a** ($R = CN$) showed high potency against HONE1 and MCF cell lines while **20b** ($R = COOEt$) showed high potency towards all the cancer cell lines except with HEPG2 that showed low potency. In addition, compound **20b** showed cytotoxicity against HA22T cell line very close to that of foretinib with IC_{50} 56 nM. The cytotoxicity evaluation of the synthesized compounds toward the six cancer cell lines are illustrated through Figure 1.

Toxicity

Bioactive compounds are often toxic to shrimp larvae. Thus, in order to monitor these chemicals in vivo lethality to shrimp larvae (*Artemiasalina*), Brine-Shrimp Lethality Assay [46] was used.

Results were analyzed with LC₅₀ program to determine LC₅₀ values and 95% confidence intervals [47]. Results are given in Table 2 the following ten compounds **5**, **7a**, **10c**, **10d**, **13b**, **14a**, **18b**, **18d**, **18e** and **20b** which exhibited optimal cytotoxic effect against cancer cell lines. The shrimp lethality assay was considered as a useful tool for preliminary assessment of toxicity, and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticides, and cytotoxicity testing of dental materials [48] natural and synthetic organic compounds [49]. It has also been shown that *A. salina* toxicity test results have a correlation with rodent and human acute oral toxicity data. Generally, a good correlation was obtained between *A. salina* toxicity test and the rodent data. Likewise, the predictive screening potential of the aquatic invertebrate tests for acute oral toxicity in man, including *A. salina* toxicity test, was slightly better than the rat test for test compounds.

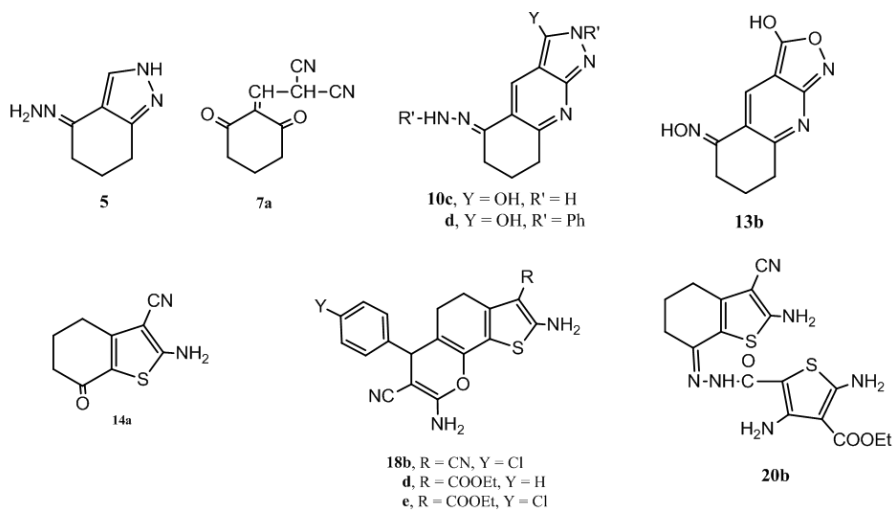
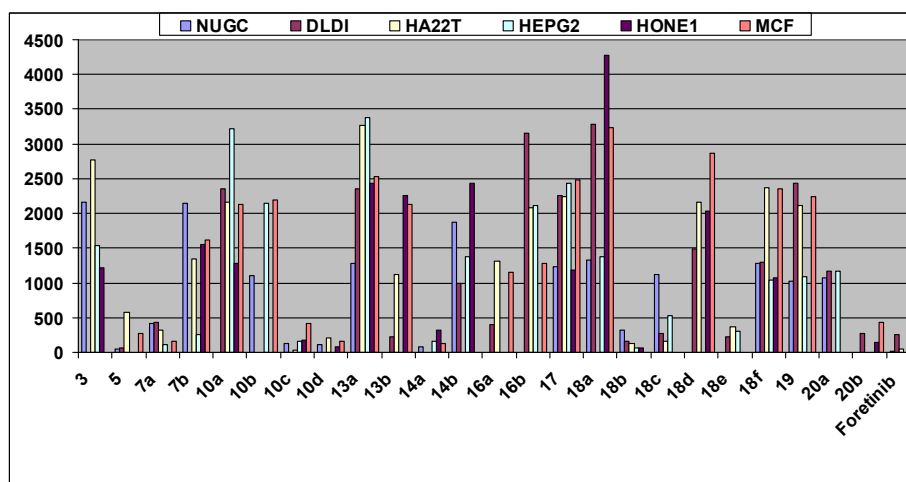


Figure 1. Structures of the most potent compounds.

In order to prevent the toxicity results from possible false effects originated from solubility of compounds and dimethyl-sulfoxide DMSO's possible toxicity effect, solutions of the test compounds were prepared in the suggested dimethyl-sulfoxide (DMSO) volume ranges. It is clear from Table 2 that compounds **7a**, **10c**, **13b**, **14a**, **18b** and **18d** were non toxic against the tested organisms. While compound **10d** was very toxic and that compounds **5**, **18e** and **20a** were harmful towards the tested organisms.

Table 2. Toxicity of the most potent compounds **5**, **7a**, **10c**, **10d**, **13b**, **14a**, **18b**, **18d**, **18e** and **20b** against shrimp larvae.

Compound No.	Conc. ($\mu\text{g}/\text{mL}$)	Mortality ^a	Toxicity	LC ₅₀	Upper 95% limit	Lower 95% limit
5	10	1	Harmful	156	120	60.5
	100	3				
	1000	8				
7a	10	0	Non toxic	993	-	-
	100	2				
	1000	3				
10c	10	0	Non toxic	980	-	-
	100	1				
	1000	4				
10d	10	4	Very toxic	273	180	142
	100	7				
	1000	10				
13b	10	0	Non toxic	970	-	-
	100	1				
	1000	2				
14a	10	0	Non toxic	874	-	-
	100	1				
	1000	3				
18b	10	0	Non toxic	969	147	93.3
	100	1				
	1000	4				
18d	10	0	Non toxic	970	-	-
	100	0				
	1000	3				
18e	10	1	Harmful	254	120	79.6
	100	2				
	1000	8				
20b	10	1	Harmful	340	89.5	60.2
	100	2				
	1000	6				

^aTen organisms (*A. salina*) tested for each concentration.

HTRF kinase assay

We studied the activity of the synthesized compounds towards PC-3 prostate cancer cell line, results are demonstrated through Table 3. The c-Met kinase activity of all compounds was evaluated using homogeneous time-resolved fluorescence (HTRF) assay as previously reported [50, 51]. Briefly, 20 $\mu\text{g}/\text{mL}$ poly (Glu, Tyr) 4:1 (Sigma) was pre-coated as a substrate in 384-well plates. Then 50 μL of 10 mM ATP (Invitrogen) solution diluted in kinase reaction buffer (50 mM HEPES, Ph 7.0, 1 M DTT, 1 M MgCl_2 , 1 M MnCl_2 , and 0.1% NaN_3) was added to each well. Various concentrations of compounds diluted in 10 mL of 1% DMSO (v/v) were used as the negative control. The kinase reaction was initiated by the addition of purified tyrosine kinase

proteins diluted in 39 mL of kinase reaction buffer solution. The incubation time for the reactions was 30 min at 25 °C and stopped by the addition of 5 mL of Streptavidin-XL665 and 5 µL Tk Antibody Cryptate working solution to all of wells. The plates were read using Envision (PerkinElmer) at 320 and 615 nM. The inhibition rate (%) was calculated using the following equation: % inhibition = 100 - [(Activity of enzyme with tested compounds - Min)/(Max - Min)] x 100 (Max: the observed enzyme activity measured in the presence of enzyme, substrates, and cofactors; Min: the observed enzyme activity in the presence of substrates, cofactors and in the absence of enzyme). IC₅₀ values were calculated from the inhibition curves.

In vitro enzymatic assays

All newly synthesized thiophene derivatives were evaluated for their inhibitory activity toward c-Met enzyme using a homogeneous time-resolved fluorescence (HTRF) assay. Taking foretinib as the positive control, the results expressed as IC₅₀ were summarized in Table 1. The IC₅₀ values are the average of at least three independent experiments. As illustrated in Table 1, all the tested compounds displayed potent c-Met enzymatic activity with IC₅₀ values ranging from 0.29 to 12.25 nM, compared with foretinib (IC₅₀ = 1.16 nM).

Table 3. c-Met enzymatic activity and anti-proliferative evaluation of target compounds against PC-3 cell line.

Compound No.	Y	R	R'	IC ₅₀ (nM) c-Met	IC ₅₀ (nM) PC-3
3	-	-	-	12.25 ± 6.17	8.29 ± 4.62
5	-	-	-	8.42 ± 3.05	0.93 ± 0.20
7a	-	CN	-	1.38 ± 0.84	0.42 ± 0.16
7b	-	COOEt	-	1.06 ± 0.37	0.63 ± 0.25
10a	NH ₂	-	H	2.46 ± 0.81	6.75 ± 1.26
10b	NH ₂	-	Ph	8.84 ± 4.58	9.36 ± 4.27
10c	OH	-	H	0.39 ± 0.14	4.26 ± 1.29
10d	OH	-	Ph	0.92 ± 0.28	1.15 ± 0.62
13a	NH ₂	-	-	8.15 ± 2.03	0.46 ± 0.17
13b	OH	-	-	1.28 ± 0.51	0.16 ± 0.02
14a	-	CN	-	3.06 ± 1.17	0.31 ± 0.05
14b	-	COOEt	-	1.23 ± 0.72	0.90 ± 0.23
16a	-	CN	-	1.23 ± 2.55	0.63 ± 0.19
16b	-	COOEt	-	1.21 ± 0.61	0.12 ± 0.21
17	-	-	-	5.32 ± 1.52	3.42 ± 1.29
18a	H	CN	-	4.56 ± 1.29	3.18 ± 1.27
18b	Cl	CN	-	0.29 ± 0.08	0.09 ± 0.002
18c	OCH ₃	CN	-	2.42 ± 1.39	0.29 ± 0.05
18d	H	COOEt	-	3.26 ± 1.42	1.35 ± 0.58
18e	Cl	COOEt	-	0.64 ± 0.21	0.28 ± 0.09
18f	OCH ₃	COOEt	-	5.43 ± 1.16	2.29 ± 0.85
19	-	-	-	0.43 ± 0.28	0.22 ± 0.06
20a	-	CN	-	4.19 ± 1.34	8.58 ± 2.41
20b	-	COOEt	-	1.05 ± 0.59	1.25 ± 0.46
	-	-	-	Foretinib 1.16 ± 0.17	SGL-1776 4.86 ± 0.16

It is clear from Table 3 that compounds **3** and **5** showed low inhibition toward c-Met but compound **5** showed high inhibition toward the prostate cancer cell line PC-3. On the other hand compounds **7a** and **7b** showed high inhibitions toward c-Met kinase and PC-3 cell line. Considering the 5,6,7,8-tetrahydro-pyrazolo[3,4-*b*]quinoline derivatives **10a-d**, it is clear that compounds **10c** (Y = OH) and **10d** (Y = OH, R' = Ph) showed the highest inhibition toward c-Met kinase among the four compounds. While compound **10d** showed high cytotoxicity toward PC-3 cell line with IC₅₀ 1.15 nM. Considering the 5,6,7,8-tetrahydroisoxazolo[3,4-*b*]quinoline derivatives **13a** and **13b** where compound **13b** (Y = OH) revealed higher inhibition toward c-Met kinase than compound **13a** (Y = NH₂). However both of **13a** and **13b** showed high inhibition toward PC-3 cell line with IC₅₀'s 0.46 and 0.16 nM, respectively. The thiophene derivative **14a** (X = CN) showed high inhibition towards PC-3 cell line with IC₅₀ 0.31 nM and it gave moderate potency against c-Met kinase but the thiophene derivative **14b** (X = COOEt) gave high potencies against both of c-Met kinase and PC-3 cell line. For the hydrazide-hydrazone derivatives **16a** and **16b** it is obvious that both of them showed high potencies against c-Met kinase and PC-3 cell line. Compound **17** showed low potencies against c-Met kinase and PC-3 cell line. For compounds **18a-f**, it is clear that compounds **18b** (X = Cl, R = CN) and **18e** (X = Cl, R = COOEt) showed high inhibition toward c-Met kinase and PC-3 cell line. The 2-cyanoacetamide derivative **19** showed high potencies c-Met kinase and PC-3 cell line with IC₅₀'s 0.43 and 0.22 nM, respectively. Considering the dihydrobenzo[*b*]thiophen-7(4*H*)-ylidene)-thiophene-2-carbohydrazone derivatives **20a,b** it is evident that compound **20b** showed highest potencies c-Met kinase and PC-3 cell line. Among the tested compounds it is clear from Table 3 that compounds **10c**, **10d**, **18b**, **18e**, **19** and **20b** showed higher potencies against c-Met kinase than the reference foretinib. While compounds **7a,7b**, **10d**, **13a**, **13b**, **14a**, **14b**, **16a**, **16b**, **17**, **18a-f**, **19** and **20** showed higher inhibition towards PC-3 cell line than the reference SGI-1776.

Table 4. Inhibition of tyrosine kinases (Enzyme IC₅₀ (nM) for compounds **5**, **7a**, **10c**, **10d**, **13b**, **14a**, **18b**, **18d**, **18e**, **20a** and **20b**.

Compound	c-Kit	Flt-3	VEGFR-2	EGFR	PDGFR
5	1.23	1.08	0.98	2.41	1.62
7a	8.27	10.40	8.59	10.28	8.63
10c	1.09	1.21	1.47	0.68	2.30
10d	0.24	0.25	0.29	0.51	0.32
13b	1.26	1.01	0.73	1.18	0.62
14a	10.21	9.32	8.07	7.59	9.23
18b	1.35	1.59	0.83	0.37	1.25
18d	0.37	0.69	0.42	0.29	0.41
18e	1.40	1.52	1.61	0.26	0.80
20a	5.27	6.21	8.39	4.18	7.33
20b	0.34	0.26	0.41	0.64	1.38
Foretinib	0.19	0.17	0.20	0.13	0.26

Inhibition of selected compounds against tyrosine kinases

The most potent compounds toward the cancer cell lines **5**, **7a**, **10c**, **10d**, **13b**, **14a**, **18b**, **18d**, **18e**, **20a** and **20b** were further investigated towards the five tyrosine kinases c-kit, FIT-3, VEGFR-2, EGFR and PDGFR and the data were expressed through Table 4. It is clear that compounds **5**, **10c**, **10d**, **13b**, **18b**, **18d**, **18e** and **20b** showed the highest inhibitory effect while compounds **7a**, **14a** and **20a** showed the least inhibition. It is of great value to note that compounds **10d**, **18d** and **20b** exhibit the maximum inhibitory effect among the eleven tested compounds. Specifically, the 5-hydrazone-5,6,7,8-tetrahydro-2*H*-pyrazolo[3,4-*b*]quinoline derivative **10c** (Y = OH, R = H) showed the highest activity toward EGFR kinase with IC₅₀ 0.68

nM, while the 3-hydroxy-7,8-dihydroisoxazolo[3,4-*b*]quinolin-5(6*H*)-one oxime (**13b**) (Y = OH) gave the highest inhibition towards VEGFR-2 and PDGFR kinase with IC₅₀'s 0.73 and 0.62 nM, respectively. Compound **18e** (Y = OCH₃, R = CN) showed its highest inhibition against EGFR and PDGFR kinases with IC₅₀'s 0.26 and 0.80 nM.

Inhibition of Pim-1 kinase for compounds 10d, 13b, 18b, 18d and 20b

Although they are frequently implicated in acute myeloid leukemia (AML) [52] Pim kinases are over expressed in many other types of hematological malignancies and solid tumors. Specifically, over expression has been identified in bladder [53], prostate [54], and head and neck cancers [55], chronic lymphocytic leukemia [56], multiple myeloma [57] and other B cell malignancies [58]. Over expression of Pim kinases was often associated with poor prognosis in each of these cancers. This relation between the Pim-1 kinase and cancer encouraged us to study the Pim-1 kinase of some compounds. Thus, the five compounds **10d**, **13b**, **18b**, **18d** and **20b** were selected for testing of their inhibition for the Pim-1 kinase (Table 5) due to their anti-proliferation activities against the cancer cell lines and their high activities against c-Met kinase. The study showed that compounds **10d** (Y = OH, R = Ph), **18b** (Y = Cl, R = CN) and **20b** (R = COOEt) showed IC₅₀'s 0.28, 0.63 and 0.59 μM, respectively. While compounds **13b** (Y = OH) and **18d** (Y = H, R = COOEt) showed no activities.

Table 5. Inhibition of Pim-1 kinase for compounds **10d**, **13b**, **18b**, **18d** and **20b**.

Compound	Inhibition ratio at 10 μM	IC ₅₀ (μM)
10d	94	0.28
13b	28	> 10
18b	84	0.63
18d	36	>10
20b	88	0.59
SGI-1776	-	0.048

It is important to note that throughout the biology sections that we used two different references, foretinib was used for the cytotoxicity of compounds and c-Met enzymatic inhibition while SGI-1776 was used for Pim-1 inhibition [59].

In summary, **10c** and **18b** were the most potent compounds beside their non toxicity against shrimp larvae and high inhibition against c-Met kinase. The *in vivo* activity estimate of compounds **10c** and **18b** represent an integration of different factors and to find differences between *in vitro* and *in vivo* responses elucidated by these compounds is hardly surprising. For that reason molecular modeling of compounds **10c** and **18b** was carried out and the results showed that these two compounds were close to foretinib concerning the π-π interaction and the binding energy score.

Table 6. Docking study data showing amino acid interactions and the hydrogen bond lengths of target compounds and foretinib on c-Met kinase enzyme.

Compound No.	Number of H-bonds	Number of π-π interactions with Phe ¹²²³	Atoms of compound forming H-bond	Amino acid residues forming H-bonds (H-bond length in Å)	Binding energy score (kcal/mol)
Ligand (Foretinib)	2	1	Quinoline N (H-acceptor) CO (H-acceptor)	Met ¹¹⁶⁰ (3.05) Lys ¹¹¹⁰ (2.89)	-16.37
10c	1	-	OH (H-acceptor)	Lys ¹¹¹⁰ (3.20)	-11.25
18b	1	1	NH ₂ of pyran (H-donor)	Glu ¹¹²⁷ (2.04)	-12.82

Docking results of compounds 10c and 18b

Compounds **10c** and **18b** were selected for molecular modeling as these two compounds were the most potent compounds beside their non toxicity against shrimp larvae and high inhibition against c-Met kinase. For each docked compound, only one pose was selected based on number of binding interactions, superposition with the original ligand, docking score and the formed H-bonds were measured. The docking results obtained from the docking study are summarized in Table 6. Figures 2-4 showed docking of the reference foretinib, compounds **10a** and **18b**, respectively.

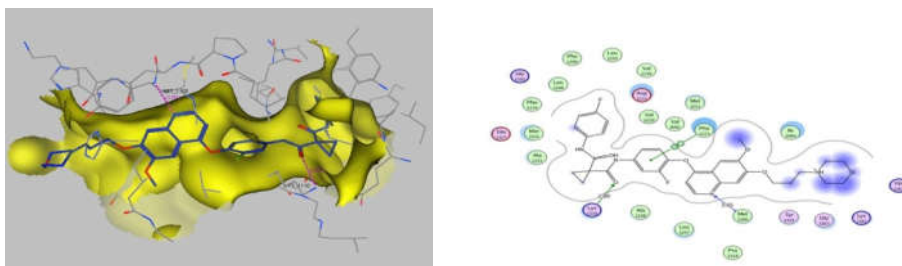


Figure 2. Interactions of XL880 (foretinib) with the amino acid residues of the active site of c-Met 3D(a) and 2D(b).

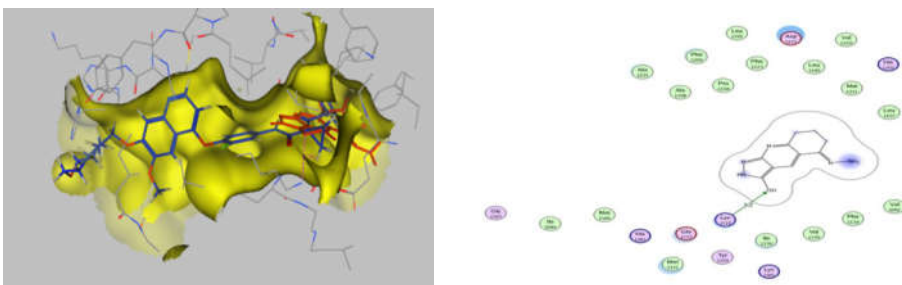


Figure 3. (a) The superposition of foretinib (blue) and compound **10c** (red) docked in the binding site of c-Met, the dotted lines represent H-bonding interactions (b) 2D ligand interaction of **10c** in binding site of c-Met.

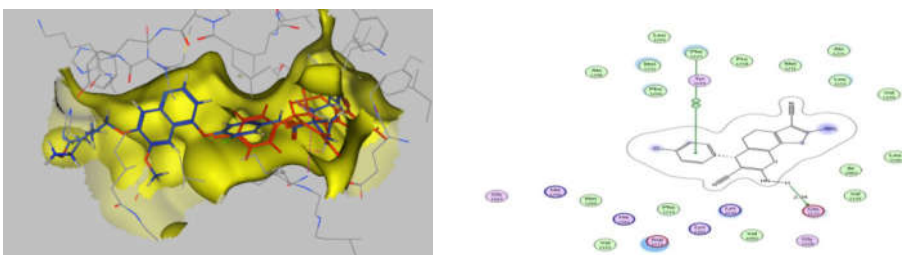


Figure 4. (a) The superposition of foretinib (blue) and compound **18b** (red) docked in the binding site of c-Met, the dotted lines represent H-bonding interactions (b) 2D ligand interaction of **18b** in binding site of c-Met.

CONCLUSION

Throughout this work the cyclohexane-1,3-dione was used for the synthesis of thiophene, pyrazole and pyran derivatives. The anti-tumor evaluation of the newly synthesized compounds against the six cancer cell NUGC, DLDI, HA22T, HEPG2, HONE1 and MCF showed that compounds **10c** and **18b** were the most potent compounds beside their non toxicity against shrimp larvae and high inhibition against c-Met kinase. The Pim-1 kinase test indicated that compounds **10d**, **18b** and **20b** were the most inhibitory compounds. It can be suggested that the inclusion of aromatic rings in the structure of the compound improves the cytotoxic activity of this class of compounds in addition, the presence of halogens, hydroxyl or cyano groups directly connected to this core also has a large contribution to the increase in this activity.

EXPERIMENTAL

Chemicals

Fetal bovine serum (FBS) and L-glutamine were purchased from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was purchased from Cambrex (New Jersey, USA). Dimethyl-sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (Saint Louis, USA).

Cell cultures

Cell cultures were obtained from the European Collection of cell Cultures (ECACC, Salisbury, UK) and human gastric cancer (NUGC and HR), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), human breast cancer (MCF), nasopharyngeal carcinoma (HONE1) and normal fibroblast cells (WI38) were kindly provided by the National Cancer Institute (NCI, Cairo, Egypt). They grow as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% heat inactivated FBS, 2 μ M glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 μ g/mL), at 37 °C in a humidified atmosphere containing 5% CO₂. Exponentially growing cells were obtained by plating 1.5 x 10⁵ cells/mL for the seven human cancer cell lines including cells derived from 0.75 x 10⁴ cells/mL followed by 24 h of incubation. The effect of the vehicle solvent DMSO on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (0.5%) of DMSO used in each assay.

The heterocyclic compounds, prepared in this study, were evaluated according to standard protocols for their in vitro cytotoxicity against seven human cancer cell lines including cells derived from human gastric cancer (NUGC and HR), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), human breast cancer (MCF), nasopharyngeal carcinoma (HONE1) and normal fibroblast cells (WI38). All of IC₅₀ values are listed in Table 1 using foretinib as the reference compound. Some heterocyclic compounds was observed with significant cytotoxicity against most of the cancer cell lines tested (IC₅₀ = 10–1000 nM). Normal fibroblasts cells (WI38) were affected to a much lesser extent (IC₅₀ > 10,000 nM).

Procedure of HTRF kinase

Briefly, 20 μ g/mL poly (Glu, Tyr) 4:1 (Sigma) was used as a substrate in 384-well plates. Then 50 μ L of 10 mM ATP (Invitrogen) solution diluted in kinase reaction buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), Ph 7.0, 1 M dithiothreitol (DTT), 1 M MgCl₂, 1 M MnCl₂, and 0.1% NaN₃) was added to each well. Various concentrations of the tested compounds diluted in 10 μ L of 1% DMSO (v/v) were used as the negative control. The

kinase reaction was started by the addition of purified tyrosine kinase proteins diluted in 39 μL of kinase reaction buffer solution. The incubation time for the reactions was 30 min at 25 $^{\circ}\text{C}$ and ceased by the addition of 5 μL of Streptavidin-XL665 and 5 μL Tk Antibody Cryptate working solution to all of wells. The plates were read using Envision (PerkinElmer) at 320 and 615 nm. The inhibition rate (%) was calculated using the mathematical equation: % inhibition = $100 - [(Activity\ of\ enzyme\ with\ tested\ compounds - Min)/(Max - Min)] \times 100$ (Max: the observed enzyme activity measured in the presence of enzyme, substrates, and cofactors; Min: the observed enzyme activity in the presence of substrates, cofactors and in the absence of enzyme).

General

All melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected. IR spectra (KBr discs) were recorded on a FTIR plus 460 or Pye Unicam SP-1000 spectrophotometer (Pye Unicam, UK, Cambridge). ^1H NMR and ^{13}C NMR spectra were recorded with Varian Gemini-200 (200 MHz, Varian UK) and JEOL AS 500 MHz (JEOL, Japan) instruments in DMSO-d_6 as solvent, using Tetraethylsilane (TMS) as internal standard chemical shifts are expressed as δ ppm. The mass spectra were recorded with Hewlett Packard 5988 A GC/MS system (Hewlett Packard, Agilent, USA) and GCMS-QP 1000Ex Shimadzu (EI, 70 eV) (Shimadzu, Japan) instruments. Analytical data were obtained from on Vario EL III Elemental CHNS analyzer. Compound **3** was previously reported, although its characterization and spectral data were not presented [31].

2-(Ethoxymethylene)cyclohexane-1,3-dione (3). To a solution of cyclohexan-1,3-dione (1.12 g, 0.01 mol) in acetic acid (40 mL) ethyl orthoformate (1.68 g, 0.01 mol) was added. The reaction mixture was heated under reflux for 2 h then evaporated in vacuum and the remaining product was triturated with ethanol and the formed solid product was collected by filtration. Yellow crystals from ethanol, yield (1.27 g, 76%), mp 282-285 $^{\circ}\text{C}$, IR (KBr) $\nu_{\text{max}} \text{ cm}^{-1}$: 2980 (CH_2), 1689, 1686 (CO), 1632 (C=C); ^1H NMR (DMSO-d_6 , 200 MHz): δ = 1.28 (t, 3H, CH_3), 1.49-1.67 (m, 2H, CH_2), 2.65-2.73 (m, 4H, 2CH_2), 3.89 (q, 2H, CH_2), 6.79 (s, 1H, CH), ^{13}C NMR (DMSO-d_6 , 75 MHz): δ 16.3 (OCH_2CH_3), 16.2, 36.8, 39.0 (3CH_2), 62.8 (OCH_2CH_3), 112.3, 158.2 (C=CH), 177.3, 179.2 (2CO). EIMS: m/z 168 [M] $^+$ (26%); analysis calcd for $\text{C}_9\text{H}_{12}\text{O}_3$ (168.19): C, 64.27; H, 7.19%. Found: C, 64.08; H, 7.33%.

4-Hydrazono-4,5,6,7-tetrahydro-2H-indazole (5). To a solution of compound **3** (1.68 g, 0.01 mol), in ethanol (40 mL) hydrazine hydrate (0.10 g, 0.02 mol) was added. The reaction mixture was heated under reflux for 3 h then poured onto ice/water containing few drops of hydrochloric acid and the formed solid product was collected by filtration. Yellow crystals from ethanol, yield (1.15 g, 77%) mp > 300 $^{\circ}\text{C}$, IR (KBr) $\nu_{\text{max}} \text{ cm}^{-1}$: 3466-3329 (NH_2 , NH), 3487, 3342 (NH_2 , NH), 2987 (CH_2), 1663, 1658 (two exocyclic C=N), 1630 (C=C); ^1H NMR (DMSO-d_6 , 200 MHz): δ = 1.46-1.69 (m, 2H, CH_2), 2.68-2.71 (m, 4H, 2CH_2), 4.23 (s, br, 2H, D_2O exchangeable, NH_2), 5.63 (s, 1H, pyrazole H-5), 8.32 (s, 1H, D_2O exchangeable, NH); ^{13}C NMR (DMSO-d_6 , 75 MHz): δ 18.6, 36.9, 39.6 (3CH_2), 143.2, 146.8 (pyrazole C-4, C-5), 180.1, 182.6 (2C=N). EIMS: m/z 150 [M] $^+$ (38%); analysis calcd for $\text{C}_7\text{H}_{10}\text{N}_4$ (150.18): C, 55.98; H, 6.71; N, 37.31%. Found: C, 56.22; H, 6.89; N, 37.08%.

General procedure for the synthesis of the cyclohexylidene)propanenitrile derivatives **7a,b**

To a solution of compound **3** (1.68 g, 0.01 mol) in 1,4-dioxane (40 mL) containing triethylamine (0.50 mL) either of malononitrile (0.66 g, 0.01 mol) or ethyl cyanoacetate (1.07, 0.01 mol) was added. The reaction mixture, in each case, was heated under reflux for 4 h then left to cool and the formed solid product was collected by filtration.

2-((2,6-Dioxocyclohexylidene)methyl)malononitrile (7a). Yellow crystals from ethanol, yield (1.46 g, 78%), mp 280-282 °C, IR (KBr) ν_{\max} cm^{-1} : 2984 (CH_2), 2223, 2220 (2CN), 1689, 1686 (CO), 1630 (C=C); ^1H NMR (DMSO- d_6 , 200 MHz): δ = 1.46-1.69 (m, 2H, CH_2), 2.65-2.70 (m, 4H, 2 CH_2), 6.80, 7.03 (2d, 2H, CH-CH); ^{13}C NMR (DMSO- d_6 , 75 MHz): δ 18.9, 37.3, 39.4 (3 CH_2), 82.3, 138.2 (CH-CH), 116.2, 116.9 (2CN), 129.8 (cyclohexane C-2), 167.8, 169.3 (2C=O). EIMS: m/z 188 $[\text{M}]^+$ (24%); analysis calcd for $\text{C}_{10}\text{H}_8\text{N}_2\text{O}_2$ (188.18): C, 63.82; H, 4.28; N, 14.89%. Found: C, 63.69; H, 4.08; N, 15.23%.

Ethyl 2-cyano-3-(2,6-dioxocyclohexylidene)propanoate (7b). Yellow crystals from ethanol, yield (1.95 g, 83%), mp 262-264 °C. IR (KBr) ν_{\max} cm^{-1} : 2989 (CH_2), 2220 (CN), 1690, 1688-1686 (3CO), 1633 (C=C); ^1H NMR (DMSO- d_6 , 200 MHz): δ = 1.13 (t, 3H, J = 7.23 Hz, CH_3), 1.43-1.67 (m, 2H, CH_2), 2.64-2.72 (m, 4H, 2 CH_2), 4.20 (q, 2H, J = 7.23 Hz, CH_2), 6.83, 7.01 (2d, 2H, CH-CH); ^{13}C NMR (DMSO- d_6 , 75 MHz): δ 16.3 (OCH_2CH_3), 18.6, 37.7, 39.8 (3 CH_2), 56.4 (OCH_2CH_3), 82.6, 138.4 (CH-CH), 116.6 (CN), 129.6 (cyclohexane C-2), 164.2, 167.4, 169.6 (3C=O). EIMS: m/z 235 $[\text{M}]^+$ (20%); analysis calcd for $\text{C}_{12}\text{H}_{13}\text{NO}_4$ (235.24): C, 61.27; H, 5.57; N, 5.95%. Found: C, 61.38; H, 5.72; N, 6.21%.

General procedure for the synthesis of the 5,6,7,8-tetrahydro-2H-pyrazolo[3,4-b]quinoline derivatives 10a-d

To a solution either of compound **7a** (1.88 g, 0.01 mol) or **7b** (2.35 g, 0.01 mol) in 1,4-dioxane (40 mL) either of hydrazine hydrate (1.0 g, 0.02 mol) or phenylhydrazine (2.16 g, 0.02 mol) was added. The reaction mixture was heated under reflux for 4 h then poured onto ice/water containing few drops of hydrochloric acid and the formed solid product was collected by filtration.

5-Hydrazono-5,6,7,8-tetrahydro-2H-pyrazolo[3,4-b]quinolin-3-amine (10a). Yellow crystals from ethanol, yield (1.64 g, 76%), mp 290-293 °C, IR (KBr) ν_{\max} cm^{-1} : 3488-3431 (2 NH_2 , NH), 2987 (CH_2), 1650 (exocyclic C=N), 1631 (C=C); ^1H NMR (DMSO- d_6 , 200 MHz): δ = 1.40-1.69 (m, 2H, CH_2), 2.64-2.75 (m, 4H, 2 CH_2), 4.82, 5.14 (2s, 4H, D_2O exchangeable, 2 NH_2), 6.80 (s, 1H, pyridine H-4), 8.29 (s, 1H, D_2O exchangeable, NH); ^{13}C NMR (DMSO- d_6 , 75 MHz): δ 19.3, 29.3, 35.2 (3 CH_2), 119.2, 122.6, 124.9, 133.1 (pyrazole C-4, C-5, pyridine C-3, C-4), 153.8, 156.9, 169.2 (3C=N). EIMS: m/z 216 $[\text{M}]^+$ (18%); analysis calcd for $\text{C}_{10}\text{H}_{12}\text{N}_6$ (216.24): C, 55.54; H, 5.59; N, 38.86%. Found: C, 55.69; H, 5.70; N, 38.92%.

2-Phenyl-5-(2-phenylhydrazono)-5,6,7,8-tetrahydro-2H-pyrazolo[3,4-b]quinolin-3-amine (10b). Orange crystals from ethanol, yield (3.16 g, 86%), mp 233-236 °C, IR (KBr) ν_{\max} cm^{-1} : 3476-3445 (NH_2 , NH), 2989 (CH_2), 1654 (exocyclic C=N), 1633 (C=C); ^1H NMR (DMSO- d_6 , 200 MHz): δ = 1.38-1.69 (m, 2H, CH_2), 2.62-2.74 (m, 4H, 2 CH_2), 4.83 (s, 2H, D_2O exchangeable, NH_2), 6.82 (s, 1H, pyridine H-4), 7.28-7.38 (m, 10H, 2 C_6H_5), 8.27 (s, 1H, D_2O exchangeable, NH); ^{13}C NMR (DMSO- d_6 , 75 MHz): δ 19.6, 29.5, 35.8 (3 CH_2), 119.6, 120.5, 122.4, 124.5, 126.9, 127.4, 128.0, 129.0, 133.2, 133.7, 134.1 (2 C_6H_5 , pyrazole C-4, C-5, pyridine C-3, C-4), 153.6, 158.3, 164.2 (3C=N). EIMS: m/z 368 $[\text{M}]^+$ (22%); analysis calcd for $\text{C}_{22}\text{H}_{20}\text{N}_6$ (368.43): C, 71.72; H, 5.47; N, 22.81%. Found: C, 71.90; H, 5.63; N, 23.01%.

5-Hydrazono-5,6,7,8-tetrahydro-2H-pyrazolo[3,4-b]quinolin-3-ol (10c). Pale yellow crystals from ethanol, yield (1.47 g, 68%), mp 210-212 °C, IR (KBr) ν_{\max} cm^{-1} : 3543-3428 (OH, NH_2 , NH), 2986 (CH_2), 1658 (exocyclic C=N), 1630 (C=C); ^1H NMR (DMSO- d_6 , 200 MHz): δ = 1.35-1.68 (m, 2H, CH_2), 2.60-2.76 (m, 4H, 2 CH_2), 4.86 (s, 2H, NH_2), 6.86 (s, 1H, pyridine H-4), 8.26 (s, 1H, D_2O exchangeable, NH), 10.20 (s, 1H, D_2O exchangeable, OH); ^{13}C NMR (DMSO- d_6 , 75 MHz): δ 19.8, 29.9, 35.5 (3 CH_2), 119.8, 122.1, 124.7, 133.3 (pyrazole C-4, C-5, pyridine

C-3, C-4), 153.8, 158.9, 164.0 (3C=N). EIMS: m/z 217 $[M]^+$ (32%); analysis calcd for $C_{10}H_{11}N_5O$ (217.23): C, 55.29; H, 5.10; N, 32.24%. Found: C, 55.42; H, 5.23; N, 32.38%.

2-Phenyl-5-(2-phenylhydrazono)-5,6,7,8-tetrahydro-2H-pyrazolo[3,4-b]quinolin-3-ol (10d). Pale yellow crystals from ethanol, yield (2.85 g, 70%), mp 244-247 °C, IR (KBr) ν_{max} cm^{-1} : 3551-3426 (OH, NH), 2985 (CH₂), 1656 (exocyclic C=N), 1626 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.33-1.67 (m, 2H, CH₂), 2.62-2.78 (m, 4H, 2CH₂), 6.85 (s, 1H, pyridine H-4), 7.29-7.40 (m, 10H, 2C₆H₅), 8.24 (s, 1H, D₂O exchangeable, NH), 10.20 (s, 1H, D₂O exchangeable, OH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 19.4, 29.7, 35.9 (3CH₂), 119.5, 122.7, 133.0, 124.3, 124.4, 125.9, 126.7, 128.0, 129.1, 130.2, 131.6, 133.8 (2C₆H₅, pyrazole C-4, C-5, pyridine C-3, C-4), 156.6, 158.7, 163.5 (3C=N). EIMS: m/z 369 $[M]^+$ (26%); analysis calcd for $C_{22}H_{19}N_5O$ (369.42): C, 71.53; H, 5.18; N, 18.96%. Found: C, 71.38; H, 5.36; N, 19.19%.

General procedure for the synthesis of the 5,6,7,8-tetrahydroisoxazolo[3,4-b]quinoline derivatives 13a,b

To a solution either of compound **7a** (1.88 g, 0.01 mol) or **7b** (2.35 g, 0.01 mol) in 1,4-dioxane (40 mL) containing sodium acetate (1.0 g) hydroxylamine hydrochloride (0.69 g, 0.01 mol) was added. The reaction mixture was heated under reflux for 3 h then poured onto ice/water mixture and the formed solid product was collected by filtration.

3-Amino-7,8-dihydroisoxazolo[3,4-b]quinolin-5(6H)-one oxime (13a). Pale yellow crystals from ethanol, yield (1.65 g, 76%), mp 287-289 °C, IR (KBr) ν_{max} cm^{-1} : 3563-3423 (OH, NH₂), 2988 (CH₂), 1656 (exocyclic C=N), 1632 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.31-1.68 (m, 2H, CH₂), 2.63-2.78 (m, 4H, 2CH₂), 4.88 (s, 2H, D₂O exchangeable, NH₂), 6.84 (s, 1H, pyridine H-4), 10.26 (s, 1H, D₂O exchangeable, OH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 19.4, 29.7, 35.9 (3CH₂), 120.5, 124.9, 126.3, 130.5 (isoxazole C-4, C-5, pyridine C-3, C-4), 158.7, 159.3, 163.8 (3C=N). EIMS: m/z 218 $[M]^+$ (26%); analysis calcd for $C_{10}H_{10}N_4O_2$ (218.21): C, 55.04; H, 4.62; N, 25.68%. Found: C, 55.22; H, 4.80; N, 25.77%.

3-Hydroxy-7,8-dihydroisoxazolo[3,4-b]quinolin-5(6H)-one oxime (13b). Pale yellow crystals from ethanol, yield (1.75 g, 80%), mp 231-235 °C, IR (KBr) ν_{max} cm^{-1} : 3572-3438 (2OH), 2989 (CH₂), 1654 (exocyclic C=N), 1630 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.33-1.69 (m, 2H, CH₂), 2.65-2.78 (m, 4H, 2CH₂), 6.83 (s, 1H, pyridine H-4), 10.23, 10.25 (2s, 2H, D₂O exchangeable, 2OH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 19.8, 29.4, 35.7 (3CH₂), 120.5, 124.9, 126.3, 130.5 (isoxazole C-4, C-5, pyridine C-3, C-4), 158.9, 159.6, 164.4 (3C=N). EIMS: m/z 219 $[M]^+$ (20%); analysis calcd for $C_{10}H_9N_3O_3$ (219.20): C, 54.79; H, 4.14; N, 19.17%. Found: C, 54.88; H, 4.07; N, 19.32%.

General procedure for the synthesis of the 5,6-dihydrobenzo[b]thiophene derivatives 14a,b

To a solution of cyclohexan-1,3-dione (11.2 g, 0.10 mol) in 1,4-dioxane (70 mL) containing triethylamine (2.0 mL) each of elemental sulfur (3.20 g, 0.10 mol) and either of malononitrile (6.60 g, 0.10 mol) or ethyl cyanoacetate (10.70 g, 0.10 mol) were added. The whole reaction mixture was heated under reflux for 1 h then left to cool. The formed solid product, in each case, was collected by filtration.

2-Amino-7-oxo-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carbonitrile (14a). Orange crystals from ethanol, yield (1.26 g, 66%), mp 186-188 °C, IR (KBr) ν_{max} cm^{-1} : 3480-3321 (NH₂), 2986 (CH₂), 2221 (CN), 1688 (CO), 1630 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.44-1.69 (m, 2H, CH₂), 2.65-2.73 (m, 4H, 2CH₂), 4.82 (s, 2H, D₂O exchangeable, NH₂); ¹³C NMR (DMSO-d₆, 75 MHz): δ 17.2, 24.6, 32.6 (3CH₂), 116.9 (CN), 125.8, 128.3, 136.7, 142.9 (thiophene C), 167.8

(CO). EIMS: m/z 192 $[M]^+$ (34%); analysis calcd for $C_9H_8N_2OS$ (192.24): C, 56.23; H, 4.19; N, 14.57; S, 16.68%. Found: C, 56.42; H, 4.23; N, 14.80; S, 16.72%.

Ethyl 2-amino-7-oxo-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (14b). Orange crystals from ethanol, yield (1.74 g, 73%), mp 105-107 °C, IR (KBr) ν_{max} cm^{-1} : 3484-3342 (NH₂), 2988 (CH₂), 1693, 1689 (2CO), 1632 (C=C); ¹H NMR (DMSO-*d*₆, 200 MHz): δ = 1.13 (t, 3H, J = 7.22 Hz, CH₃), 1.42-1.69 (m, 2H, CH₂), 2.64-2.73 (m, 4H, 2CH₂), 4.23 (q, 2H, J = 7.22 Hz, CH₂), 4.84 (s, 2H, D₂O exchangeable, NH₂); ¹³C NMR (DMSO-*d*₆, 75 MHz): δ 16.8 (OCH₂CH₃), 17.8, 24.2, 32.1 (3CH₂), 56.3 (OCH₂CH₃), 126.3, 128.9, 134.8, 143.2 (thiophene C), 164.2, 168.3 (2CO). EIMS: m/z 239 $[M]^+$ (26%); analysis calcd for $C_{11}H_{13}NO_3S$ (239.29): C, 55.21; H, 5.48; N, 5.85; S, 13.40%. Found: C, 55.39; H, 5.52; N, 6.18; S, 13.66%.

General procedure for the synthesis of the 5,6-dihydrobenzo[b]thiophen-7(4H)-ylidene)-2-cyanoacetohydrazide 16a,b

Method (A). Equimolar amounts of either of compound **14a** (1.92 g, 0.01 mol) or **14b** (2.39 g, 0.01 mol) and cyanoacetylhydrazine (1.0 g, 0.01 mol) in 1,4-dioxane (40 mL) was heated under reflux for 2 h then left to cool. The formed solid product, in each case was collected by filtration.

Method (B). To a solution of compound **17** (1.93 g, 0.01 mol) in 1,4-dioxane (70 mL) containing triethylamine (2.0 mL) each of elemental sulfur (0.32 g, 0.01 mol) and either of malononitrile (6.60 g, 0.10 mol) or ethyl cyanoacetate (1.07 g, 0.01 mol) were added. The whole reaction mixture was heated under reflux for 1 h then left to cool. The formed solid product, in each case, was collected by filtration.

N'-(2-Amino-3-cyano-5,6-dihydrobenzo[b]thiophen-7(4H)-ylidene)-2-cyanoacetohydrazide (16a). Orange crystals from ethanol, yield (1.85 g, 68%), mp 215-218 °C, IR (KBr) ν_{max} cm^{-1} : 3489-3327 (NH₂, NH), 2980 (CH₂), 2260 (CN), 1688 (CO), 1630 (C=C); ¹H NMR (DMSO-*d*₆, 200 MHz): δ = 1.40-1.68 (m, 2H, CH₂), 2.60-2.71 (m, 4H, 2CH₂), 4.42 (s, 2H, CH₂), 4.80 (s, 2H, D₂O exchangeable, NH₂), 8.31 (s, 1H, D₂O exchangeable, NH); ¹³C NMR (DMSO-*d*₆, 75 MHz): δ 18.3, 24.6, 32.8 (3CH₂), 58.8 (CH₂), 116.6, 117.4 (2CN), 126.6, 128.7, 134.6, 143.8 (thiophene C), 164.8 (CO), 172.3 (C=N). EIMS: m/z 273 $[M]^+$ (18%); analysis calcd for $C_{12}H_{11}N_5OS$ (273.31): C, 52.73; H, 4.06; N, 25.62; S, 11.73%. Found: C, 52.49; H, 4.32; N, 25.80; S, 11.84%.

Ethyl 2-amino-7-(2-(2-cyanoacetyl)hydrazono)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (16b). Orange crystals from ethanol, yield (2.62 g, 82%), mp 147-149 °C, IR (KBr) ν_{max} cm^{-1} : 3459-3342 (NH₂, NH), 2983 (CH₂), 1689, 1686 (2CO), 1654 (exocyclic C=N), 1632 (C=C); ¹H NMR (DMSO-*d*₆, 200 MHz): δ = 1.13 (t, 3H, J = 7.04 Hz, CH₃), 1.42-1.69 (m, 2H, CH₂), 2.63-2.70 (m, 4H, 2CH₂), 4.22 (q, 2H, J = 7.04 Hz, CH₂), 4.40 (s, 2H, CH₂), 4.81 (s, 2H, D₂O exchangeable, NH₂), 8.32 (s, 1H, D₂O exchangeable, NH); ¹³C NMR (DMSO-*d*₆, 75 MHz): δ 16.9 (OCH₂CH₃), 18.4, 24.2, 32.9 (3CH₂), 56.2 (OCH₂CH₃), 116.4 (CN), 126.4, 128.3, 134.8, 143.8 (thiophene C), 164.9, 166.5 (2CO). EIMS: m/z 320 $[M]^+$ (25%); analysis calcd for $C_{14}H_{16}N_4O_3S$ (320.37): C, 52.49; H, 5.03; N, 17.49; S, 10.01%. Found: C, 52.53; H, 4.92; N, 17.28; S, 10.32%.

2-Cyano-N'-(3-oxocyclohexylidene)acetohydrazide (17). To a solution of cyclohexan-1,3-dione (1.12 g, 0.01 mol) in 1,4-dioxane (40 mL) cyanoacetylhydrazine (1.0 g, 0.01 mol) was added. The reaction mixture was heated under reflux for 2 h then left to cool. The formed solid product, in each case was collected by filtration. White crystals from ethanol, yield (1.5 g, 78%), mp 220-222 °C, IR (KBr) ν_{max} cm^{-1} : 3473-3330 (NH), 2984 (CH₂), 1691, 1687 (2CO), 1650

(exocyclic C=N), 1630 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.43-1.69 (m, 2H, CH₂), 2.62-2.73 (m, 4H, 2CH₂), 2.93 (s, 2H, CH₂), 4.43 (s, 2H, CH₂), 8.29 (s, 1H, D₂O exchangeable, NH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 18.5, 26.3, 34.2, 38.4 (4CH₂), 64.2 (CH₂), 117.2 (CN), 126.4, 128.3, 134.8, 143.8 (thiophene C), 164.6, 167.2 (2CO), 172.4 (C=N). EIMS: m/z 193 [M]⁺ (32%); analysis calcd for C₉H₁₁N₃O₂ (193.20): C, 55.95; H, 5.74; N, 21.75%. Found: C, 55.69; H, 5.92; N, 21.80%.

General procedure for the synthesis of the 5,6-dihydro-4H-thieno[3,2-h]chromene derivatives 18a-f

To a solution of either of compound **14a** (2.86 g, 0.01 mol), **14b** (3.20 g, 0.01 mol) in 1,4-dioxane (40 mL) containing triethylamine (0.50 mL) any of benzaldehyde (1.06 g, 0.01 mol), 4-chlorobenzaldehyde (1.40 g, 0.01 mol) or 4-methoxybenzaldehyde (1.83 g, 0.01 mol) and malononitrile (0.66 g, 0.01 mol) were added. The whole reaction mixture was heated under reflux for 3 h then left to cool and the formed solid product, in each case, was collected by filtration.

2,8-Diamino-4-phenyl-5,6-dihydro-4H-thieno[3,2-h]chromene-3,7-dicarbonitrile (18a). Yellow crystals from 1,4-dioxane, yield (2.42 g, 70%), mp 130-132 °C, IR (KBr) ν_{max} cm⁻¹: 3489-3341 (NH₂), 2980 (CH₂), 2223, 2220 (2CN), 1626 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 2.62-2.78 (2t, 4H, J = 6.87 Hz, 2CH₂), 4.47, 4.80 (2s, 4H, D₂O exchangeable, 2NH₂), 6.09 (s, 1H, pyran H-4), 7.28-7.41 (m, 5H, C₆H₅); ¹³C NMR (DMSO-d₆, 75 MHz): δ 44.6, 50.3 (2CH₂), 60.8 (pyran C-4), 116.8, 117.2 (2CN), 120.3, 122.1, 125.8, 126.4, 128.9, 129.2, 129.6, 132.8, 133.1, 136.5, 143.8, 145.7, 150.6 (C₆H₅, pyran, thiophene C). EIMS: m/z 346 [M]⁺ (28%); analysis calcd for C₁₉H₁₄N₄OS (346.41): C, 65.88; H, 4.07; N, 16.17; S, 9.26%. Found: C, 65.69; H, 3.90; N, 16.08; S, 9.42%.

2,8-Diamino-4-(4-chlorophenyl)-5,6-dihydro-4H-thieno[3,2-h]chromene-3,7-dicarbonitrile (18b). Yellow crystals from 1,4-dioxane, yield (2.74 g, 72%), mp 102-104 °C, IR (KBr) ν_{max} cm⁻¹: 3465-3328 (NH₂), 2983 (CH₂), 2223, 2220 (2CN), 1628 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 2.64-2.79 (2t, 4H, 2CH₂), 4.48, 4.82 (2s, 4H, D₂O exchangeable, 2NH₂), 6.09 (s, 1H, pyran H-4), 7.24-7.47 (m, 4H, C₆H₄); ¹³C NMR (DMSO-d₆, 75 MHz): δ 44.9, 50.1 (2CH₂), 60.6 (pyran C-4), 116.7, 117.5 (2CN), 120.6, 122.3, 125.4, 126.8, 128.2, 129.0, 131.2, 132.6, 133.6, 143.8, 145.5, 150.8 (C₆H₄, pyran, thiophene C). EIMS: m/z 380 [M]⁺ (46%); analysis calcd for C₁₉H₁₃ClN₄OS (380.85): C, 59.92; H, 3.44; N, 14.71; S, 8.42%. Found: C, 60.16; H, 3.52; N, 14.93; S, 8.66%.

2,8-Diamino-4-(4-methoxyphenyl)-5,6-dihydro-4H-thieno[3,2-h]chromene-3,7-dicarbonitrile (18c). Yellow crystals from 1,4-dioxane, yield (2.25 g, 60%), mp 182-185 °C, IR (KBr) ν_{max} cm⁻¹: 3485-3341 (NH₂), 2980, 2873 (CH₃, CH₂), 2224, 2220 (2CN), 1629 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 2.62-2.75 (2t, 4H, 2CH₂), 3.69 (s, 3H, OCH₃), 4.42, 4.86 (2s, 4H, 2NH₂), 6.07 (s, 1H, pyran H-4), 7.26-7.49 (m, 4H, C₆H₄); ¹³C NMR (DMSO-d₆, 75 MHz): δ 44.8, 50.6 (2CH₂), 53.6 (OCH₃), 60.3 (pyran C-4), 116.9, 117.0 (2CN), 121.8, 122.6, 125.4, 127.3, 128.0, 130.4, 132.5, 133.3, 136.6, 143.7, 145.3, 150.2 (C₆H₅, pyran, thiophene C). EIMS: m/z 376 [M]⁺ (26%); analysis calcd for C₂₀H₁₆N₄O₂S (376.43): C, 63.81; H, 4.28; N, 14.88; S, 8.52%. Found: C, 64.01; H, 4.37; N, 14.59; S, 8.71%.

Ethyl 2,8-diamino-3-cyano-4-phenyl-5,6-dihydro-4H-thieno[3,2-h]chromene-7-carboxylate (18d). Pale yellow crystals from 1,4-dioxane, yield (2.67 g, 68%), mp 194-196 °C, IR (KBr) ν_{max} cm⁻¹: 3480-3358 (NH₂), 2986, 2878 (CH₃, CH₂), 2220 (CN), 1688 (CO), 1627 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.14 (t, 3H, J = 6.78 Hz, CH₃), 2.60-2.78 (2t, 4H, 2CH₂), 4.22

(q, 2H, $J = 6.78$ Hz, CH₂), 4.47, 4.88 (2s, 4H, 2NH₂), 6.079 (s, 1H, pyran H-4), 7.29-7.42 (m, 5H, C₆H₅); ¹³C NMR (DMSO-d₆, 75 MHz): δ 16.8 (OCH₂CH₃), 44.5, 50.8 (2CH₂), 52.8 (OCH₂CH₃), 60.3 (pyran C-4), 116.6 (CN), 120.3, 122.5, 125.2, 126.7, 128.3, 130.6, 131.3, 134.9, 135.2, 143.9, 147.8, 150.0 (C₆H₅, pyran, thiophene C), 164.3 (CO). EIMS: m/z 393 [M]⁺ (38%); analysis calcd for C₂₁H₁₉N₃O₃S (393.46): C, 64.10; H, 4.87; N, 10.68; S, 8.15%. Found: C, 64.26; H, 4.69; N, 10.49; S, 8.65%.

Ethyl 2,8-diamino-4-(4-chlorophenyl)-3-cyano-5,6-dihydro-4H-thieno[3,2-h]chromene-7-carboxylate (18e). Yellow crystals from 1,4-dioxane, yield (3.12 g, 73%), mp 190-193 °C, IR (KBr) ν_{\max} cm⁻¹: 3489-3342 (NH₂), 2986, 2875 (CH₃, CH₂), 2222 (CN), 1680 (CO), 1625 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.14 (t, 3H, $J = 6.99$ Hz, CH₃), 2.62-2.79 (2t, 4H, 2CH₂), 4.24 (q, 2H, $J = 6.99$ Hz, CH₂), 4.47, 4.89 (2s, 4H, 2NH₂), 6.08 (s, 1H, pyran H-4), 7.24-7.49 (m, 4H, C₆H₄); ¹³C NMR (DMSO-d₆, 75 MHz): δ 16.9 (OCH₂CH₃), 44.5, 50.6 (2CH₂), 52.6 (OCH₂CH₃), 60.8 (pyran C-4), 116.9 (CN), 120.6, 122.3, 125.8, 126.9, 128.3, 130.8, 132.6, 134.6, 135.8, 146.1, 147.5, 150.3 (C₆H₅, pyran, thiophene C), 164.2 (CO). EIMS: m/z 427 [M]⁺ (30%); analysis calcd for C₂₁H₁₈ClN₃O₃S (427.90): C, 58.94; H, 4.24; N, 9.82; S, 7.49%. Found: C, 58.83; H, 4.53; N, 10.05; S, 7.66%.

Ethyl 2,8-diamino-3-cyano-4-(4-methoxyphenyl)-5,6-dihydro-4H-thieno[3,2-h]chromene-7-carboxylate (18f). Yellow crystals from 1,4-dioxane, yield (3.30 g, 78%), mp 92-94 °C, IR (KBr) ν_{\max} cm⁻¹: 3477-3328 (NH₂), 2984, 2878 (CH₃, CH₂), 2220 (CN), 1686 (CO), 1627 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.12 (t, 3H, $J = 7.31$ Hz, CH₃), 2.60-2.78 (2t, 4H, 2CH₂), 3.68 (s, 3H, OCH₃), 4.23 (q, 2H, $J = 7.31$ Hz, CH₂), 4.47, 4.87 (2s, 4H, 2NH₂), 6.06 (s, 1H, pyran H-4), 7.22-7.48 (m, 4H, C₆H₄); ¹³C NMR (DMSO-d₆, 75 MHz): δ 16.3 (OCH₂CH₃), 44.8, 50.6 (2CH₂), 52.7 (OCH₂CH₃), 53.4 (OCH₃), 60.8 (pyran C-4), 116.6 (CN), 121.2, 122.6, 125.4, 126.2, 127.8, 129.4, 132.8, 134.6, 135.8, 146.4, 147.7, 150.6 (C₆H₅, pyran, thiophene C), 164.8 (CO). EIMS: m/z 423 [M]⁺ (26%); analysis calcd for C₂₂H₂₁N₃O₄S (423.48): C, 62.40; H, 5.00; N, 9.92; S, 7.57%. Found: C, 62.59; H, 4.94; N, 10.24; S, 7.72%.

N-(2-Amino-3,7-dicyano-4-phenyl-5,6-dihydro-4H-thieno[3,2-h]chromen-8-yl)-2-cyanoacetamide (19). A solution of equimolecular amounts of compound **18a** (3.46 g, 0.01 mol) and ethyl cyanoacetate (1.07 g, 0.01 mol) in dimethylformamide (30 mL) was heated under reflux for 3 h. The solid product, so formed, upon pouring onto ice/water mixture was collected by filtration. Pale yellow crystals from 1,4-dioxane, yield (2.72 g, 66%), mp 181-183 °C, IR (KBr) ν_{\max} cm⁻¹: 3495-3345 (NH₂), 2986 (CH₂), 2223-2220 (3CN), 1687 (C=O), 1627 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.14 (t, 3H, $J = 6.99$ Hz, CH₃), 2.64-2.85 (2t, 4H, 2CH₂), 3.93 (s, 2H, CH₂), 4.89 (s, 2H, D₂O exchangeable, NH₂), 6.08 (s, 1H, pyran H-4), 7.28-7.38 (m, 5H, C₆H₅), 8.30 (s, 1H, D₂O exchangeable, NH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 44.3, 50.9 (2CH₂), 57.6 (CH₂), 60.4 (pyran C-4), 116.8, 117.1, 117.6 (3CN), 120.8, 121.9, 123.6, 124.9, 126.2, 129.4, 132.8, 134.6, 138.2, 146.4, 148.2, 149.8 (C₆H₅, pyran, thiophene C), 168.3 (CO). EIMS: m/z 413 [M]⁺ (18%); analysis calcd for C₂₂H₁₅N₅O₂S (413.45): C, 63.91; H, 3.66; N, 16.94; S, 7.76%. Found: C, 64.25; H, 3.80; N, 17.28; S, 7.82%.

General procedure for the synthesis of the dihydrobenzo[b]thiophen-7(4H)-ylidene-thiophene-2-carbohydrazide derivatives 20a,b

To a solution of compound **16a** (2.73 g, 0.01 mol) in 1,4-dioxane (40 mL) containing triethylamine (2.0 mL) each of elemental sulfur (0.32 g, 0.01 mol) and either of malononitrile (0.66 g, 0.01 mol) or ethyl cyanoacetate (1.07 g, 0.10 mol) were added. The whole reaction mixture was heated under reflux for 2 h then left to cool. The formed solid product, in each case, was collected by filtration.

3,5-Diamino-N'-(2-amino-3-cyano-5,6-dihydrobenzo[b]thiophen-7(4H)-ylidene)-4-cyanothiophene-2-carbohydrazide (20a). Orange crystals from acetic acid, yield (2.71 g, 73%), mp 182-184 °C, IR (KBr) ν_{\max} cm^{-1} : 3485-3371 (NH₂, NH), 2988 (CH₂), 2226, 2220 (2CN), 1688 (C=O), 1629 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.48 (m, 4H, 2CH₂), 2.62-2.87 (m, 2H, CH₂), 4.59, 4.89, 5.11 (3s, 6H, D₂O exchangeable, 3NH₂), 8.33 (s, 1H, D₂O exchangeable, NH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 18.8, 24.8, 35.2 (2CH₂), 116.6, 117.2 (2CN), 120.4, 122.9, 126.0, 128.2, 130.4, 136.4, 144.0, 148.2 (two thiophene C), 165.8 (CO). EIMS: m/z 371 [M]⁺ (20%); analysis calcd for C₁₅H₁₃N₇O₂ (371.44): C, 48.50; H, 3.53; N, 26.40; S, 17.27%. Found: C, 48.59; H, 3.38; N, 26.49; S, 16.91%.

Ethyl 2,4-diamino-5-(2-(2-amino-3-cyano-5,6-dihydrobenzo[b]thiophen-7(4H)-ylidene)hydrazinecarboxyl)thiophene-3-carboxylate (20b). Orange crystals from acetic acid, yield (2.92 g, 70%), mp 196-199 °C, IR (KBr) ν_{\max} cm^{-1} : 3489-3369 (NH₂, NH), 2987 (CH₂), 2220 (CN), 1687 (C=O), 1628 (C=C); ¹H NMR (DMSO-d₆, 200 MHz): δ = 1.14 (t, 3H, J = 6.55 Hz, ester CH₃), 1.49 (m, 4H, 2CH₂), 2.60-2.87 (m, 2H, CH₂), 4.23 (q, 2H, J = 6.55 Hz, ester CH₂), 4.63, 4.85, 5.13 (3s, 6H, D₂O exchangeable, 3NH₂), 8.32 (s, 1H, D₂O exchangeable, NH); ¹³C NMR (DMSO-d₆, 75 MHz): δ 16.6 (OCH₂CH₃), 18.6, 24.8, 32.9 (2CH₂), 52.6 (OCH₂CH₃), 116.8 (CN), 126.2, 128.5, 132.8, 134.6, 136.6, 146.4, 147.3, 149.4 (thiophene C), 164.2, 168.8 (2CO). EIMS: m/z 418 [M]⁺ (37%); analysis calcd for C₁₇H₁₈N₆O₃S₂ (418.49): C, 48.79; H, 4.34; N, 20.08; S, 15.32%. Found: C, 48.64; H, 4.59; N, 20.16; S, 15.52%.

ACKNOWLEDGEMENT

R. M. Mohareb would like to thank the Alexander von Humboldt Foundation in Bonn, Germany, for sponsoring this research and regularly providing visiting fellowships to Germany in order to complete the experimental work.

REFERENCES

1. Chung, M.K.; Han, S.S.; Kim, J.C. Evaluation of the toxic potentials of a new camptothecin anticancer agent CKD-602 on fertility and early embryonic development in rats. *Reg. Toxicol. Pharm.* **2006**, *45*, 273-281.
2. Ruiz, A.D.; Müller, A.R.; Ramirez, L.R.; Howarth, J.A.; Dobrota, M. Hematotoxicity response in rats by the novel copper-based anticancer agent: casiopeina II. *Toxicology* **2003**, *194*, 103-113.
3. Sanyal, U.; Nanda, R.; Samanta, S.; Pain, A.; Dutta, S.; Verma, A.S.; Rider, B.J.; Agrawal, K.C. Evaluation of dimethylaminosulfonates of alkane diols as a novel group of anticancer agents. *Cancer Lett.* **2000**, *155*, 89-97.
4. Bandgar, B.P.; Gawande, S.S. Synthesis and biological screening of a combinatorial library of β -chlorovinyl chalcones as anticancer, anti-inflammatory and antimicrobial agents. *Bioorg. Med. Chem.* **2010**, *18*, 2060-2065.
5. Banik, B.K.; Becker, F.F. Novel 6,12-disubstituted chrysene as potent anticancer agent: Synthesis, in vitro and in vivo study. *Eur. J. Med. Chem.* **2010**, *45*, 4687-4691.
6. Strohl, W.R. The role of natural products in a modern drug discovery program. *Drug Disc. Today* **2000**, *5*, 39-41.
7. Mishra, B.B.; Tiwari, V.K. Natural products: an evolving role in future drug discovery. *Eur. J. Med. Chem. Nat. Prod.* **2000**, *2*, 39-41.
8. Ibrahim, R.A. Uses of acetoacetanilide for the synthesis of thiophene derivatives with cytotoxic activities. *Bull. Chem. Soc. Ethiop.* **2017**, *31*, 519-534.

9. Valluri, K.K.; Viswanath, I.V.K.; Raju P.V.V.; Rajasekher B.; Dasu, B.R. Synthesis and in-vitro studies of some new quinoline 1,3,4-thiadiazolo pyrimidin derivatives. *Bull. Chem. Soc. Ethiop.* **2017**, *31*, 337-344.
10. Kumar, D.; Patel, G.; Johnson, E.O.; Shah, K. Synthesis and anticancer activities of novel 3,5-disubstituted-1,2,4-oxadiazoles. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2739-2741.
11. Pomarnacka, E.; Kornicka, A. Synthesis and in vitro anticancer and anti-HIV evaluation of new 2-mercaptobenzenesulfonamides. *Farmaco II* **2001**, *56*, 571-577.
12. Kaufmann, D.; Pojarová, M.; Vogel, S.; Liebl, R.; Gastpar, R.; Gross, D.; Nishino, T.; Pfaller, T.; Angerer, E. Antimitotic activities of 2-phenylindole-3-carbaldehydes in human breast cancer cells. *Bioorg. Med. Chem.* **2007**, *15*, 5122-5136.
13. Jackson, P.F.; Cole, D.C.; Slusher, B.S.; Stetz, S.L.; Ross, L.E.; Donzanti, B.A.; Trainor, D.A. Design, synthesis, and biological activity of a potent inhibitor of the neuropeptidase N-acetylated α -linked acidic dipeptidase. *J. Med. Chem.* **1996**, *39*, 619-622.
14. Omar, S.H.; Al-Wabel, N.A. Organosulfur compounds and possible mechanism of garlic in cancer. *Saudi Pharm. J.* **2010**, *18*, 51-58.
15. Keri, R.S.; Chand, K.; Budagumpi, S.; Somappa, B.S.; Nagaraja, B.M. An overview of benzo[b]thiophene-based medicinal chemistry. *Eur. J. Med. Chem.* **2017**, *138*, 1002-1033.
16. Zhang, W.; Ma, T.; Li, S.; Yang, Y.; Guo, J.; Yu, W.; Kong, L. Antagonizing STAT3 activation with benzo[b]thiophene-1,1-dioxide based small molecules. *Eur. J. Med. Chem.* **2017**, *125*, 538-550.
17. Xu, Y.Z.; Yuan, S.; Bowers, S.; Hom, R.K.; Chan, W.; Sham, H.L.; Zhu, Y.L.; Beroza, P.; Pan, H.; Brecht, E.; Yao, N.; Loughheed, J.; Yan, J.; Tam, D.; Ren, Z.; Ruslim, L.; Bova, M. P.; Artis, D.R. Design and synthesis of thiophene dihydroisoquinolines as novel BACE1 inhibitors. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 3075-3080.
18. Al-Said, M.S.; Bashandy, M.S.; Al-qasoumi, S.I.; Ghorab, M.M. Anti-breast cancer activity of some novel 1,2-dihydropyridine, thiophene and thiazole derivatives. *Eur. J. Med. Chem.* **2011**, *46*, 137-141.
19. Wang, M.; Xu, S.; Lei, H.; Wang, C.; Xiao, Z.; Jia, S.; Zhi, J.; Zheng, P.; Zhu, W. Design, synthesis and antitumor activity of novel sorafenib derivatives bearing pyrazole scaffold. *Bioorg. Med. Chem.* **2017**, *25*, 5754-5763.
20. Hu, L.; Zheng, Y.; Li, Z.; Wang, Y.; Lv, Y.; Qin, X.; Zeng, C. Design, synthesis, and biological activity of phenyl-pyrazole derivatives as BCR-ABL kinase inhibitors. *Bioorg. Med. Chem.* **2015**, *23*, 3147-3152.
21. Azzam, R.A.; Mohareb, R.M. Multicomponent reactions of acetoacetanilide derivatives with aromatic aldehydes and cyanomethylene reagents to produce 4H-pyran and 1,4-dihydropyridine derivatives with antitumor activities. *Chem. Pharm. Bull.* **2015**, *63*, 1055-1064.
22. Mohareb, R.M.; Wardakhan, W.W.; Ibrahim, R.A. Synthesis of pyridine, pyran and thiazole containing thiophene derivatives and their anti-tumor evaluations. *Med. Chem. Res.* **2016**, *25*, 2187-2204.
23. Abdallah, A.M.; Mohareb, R.M.; Khalil, E.M.; Elshamy, M.A.M. Synthesis of novel heterocyclic compounds incorporate 4,5,6,7-tetrahydrobenzo[b]thiophene together with their cytotoxic evaluations. *Chem. Pharm. Bull.* **2017**, *65*, 469-477.
24. Raffa, D.; Maggio, B.; Raimondi, M.V.; Plescia, S.C.F.; Cancemi, G.; Daidone, G.; Recent advanced in bioactive systems containing pyrazole fused with a five membered heterocycle. *Eur. J. Med. Chem.* **2015**, *97*, 732-746.
25. Prabhudeva, M.G.; Bharath, S.; Kumar, A.D.; Naveen, S.; Lokanath, N.K.; Mylarappa, B.N.; Kumar, K.A. Design and environmentally benign synthesis of novel thiophene appended pyrazole analogues as anti-inflammatory and radical scavenging agents: Crystallographic, in silico modeling, docking and SAR characterization. *Bioorg. Chem.* **2017**, *73*, 109-120.

26. Shu, S.; Cai, X.; Li, J.; Feng, Y.; Dai, A.; Wang, J.; Yang, D.; Wang, M.W.; Liu, H. Design, synthesis, structure–activity relationships, and docking studies of pyrazole-containing derivatives as a novel series of potent glucagon receptor antagonists. *Bioorg. Med. Chem.* **2016**, *24*, 2852-2863.
27. Jacominib, A.P.; Silva, M.J.V.; Silva, R.G.M.; Gonçalves, D.S.; Volpato, H.; Basso, E.A.; Paula, F.R.; Nakamura, C.V.; Sarragiotto, M.H.; Rosa, F.A. Synthesis and evaluation against *Leishmania amazonensis* of novel pyrazolo[3,4-*d*]pyridazinone-N-acylhydrazone-(π)thiophene hybrids. *Eur. J. Med. Chem.* **2016**, *124*, 340-349.
28. Abdel-Rahman, S.A.; El-Gohary, N.S.; El-Bendary, E.R.; El-Ashry, S.M.; Shaaban, M.I. Synthesis, antimicrobial, anti-quorum-sensing, antitumor and cytotoxic activities of new series of cyclopenta(hepta)[*b*]thiophene and fused cyclohepta[*b*]thiophene analogs. *Eur. J. Med. Chem.* **2017**, *140*, 200-211.
29. Ambethkar, S.; Padmini, V.; Bhuvanesh, N. A green and efficient protocol for the synthesis of dihydropyrano[2,3-*c*]pyrazole derivatives via a one-pot, four component reaction by grinding method. *J. Adv. Res.* **2015**, *6*, 975-985.
30. Mabkhot, Y.N.; Alatibi, F.; El-Sayed, N.N.E.; Al-Showiman, S.S.; Kheder, N.; Hazem, A.; Ghabbour, H.A. A facile synthesis and characterization of some new thiophene based heterocycles. *J. Mol. Struct.* **2017**, *1149*, 736-743.
31. Horst, J.; Joachim, E.H.; Otto, M.K.; Jochen, S.H.; Jaime, P.; Barcelona, E.S. *German Patent* **1985**, DE3406329 A1.
32. McKibben, B.P.; Cartwright, C.H.; Castelhana, A.L. Practical synthesis of tetrasubstituted thiophenes for use in compound libraries. *Tetrahedron Lett.* **1999**, *40*, 5471-5474.
33. Wang, K.; Kim, D.; Dömling, A. Cyanoacetamide MCR (III): Three-component Gewald reactions revisited. *J. Comb. Chem.* **2010**, *12*, 111-118.
34. Mishra, R.; Jha, K.K.; Kumar, S.; Tomer, I. Synthesis, properties and biological activity of thiophene: A review. *Der Pharma Chemica* **2011**, *3*, 38-54.
35. Xu, X.; Shi, W.; Zhou, Y.; Wang, Y.; Zhang, M.; Song, L.; Deng, H. Convenient one-pot synthesis of monofluorinated functionalized 4-*H*-pyran derivatives via multi-component reactions. *J. Fluorine Chem.* **2015**, *176*, 127-133.
36. Penta, S.; Gadidasu, K.K.; Basavoju, S.; Rao, V.R. An efficient one-pot synthesis of pyrazolyl-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-pyran-2-one derivatives via multi-component approach and their potential antimicrobial and nematocidal activities *Tetrahedron Lett.* **2013**, *54*, 5663-5666.
37. Piltan, M.J.; Ghomi J.S. Nano crystalline ZnO catalyzed one pot three-component synthesis of 7-alkyl-6*H*,7*H*-naphtho[1',2':5,6]pyrano[3,2-*c*]chromen-6-ones under solvent-free conditions. *Bull. Chem. Soc. Ethiop.* **2016**, *30*, 289-296.
38. Hazeri, N.; Maghsoudlou, M.T.; Mir, F.; Kangani, M.; Saravani, H.; Molashahi, E. An efficient one-pot three-component synthesis of tetrahydrobenzo[*b*]pyran and 3,4-dihydropyrano-*c*]chromene derivatives using starch solution as catalyst. *Chin. J. Catal.* **2014**, *35*, 391-395.
39. Wagh Y.B.; Tayade, Y.A.; Padvi, S.A.; Patil, B.S.; Patil, N.B.; Dalal, D.S. A cesium fluoride promoted efficient and rapid multicomponent synthesis of functionalized 2-amino-3-cyano-4*H*-pyran and spirooxindole derivatives. *Chin. Chem. Lett.* **2015**, *26*, 1273-1277.
40. Jolodar, O.G.; Shirini, F.; Seddighi, M. Introduction of a novel basic ionic liquid containing dual basic functional groups for the efficient synthesis of spiro-4*H*-pyrans. *J. Mol. Liq.* **2016**, *224*, 1092-1111.
41. Zakeri, M.; Nasef, M.M.; Lotf, E.A.; Moharami, A.; Heravi, M.M. Sustainable alternative protocols for the multicomponent synthesis of spiro-4*H*-pyrans catalyzed by 4-dimethylaminopyridine. *J. Induct. Engin. Chem.* **2015**, *29*, 273-28.
42. Tabassum, S.; Govindaraju, S.; Khan, R.R.; Pasha, M.A. Ultrasound mediated, iodine catalyzed green synthesis of novel 2-amino-3-cyano-4*H*-pyran derivatives. *Ultrasonic Sonochem.* **2015**, *24*, 1-7.

43. El-Sayed, N.N.E.; Abdelaziz, M.A.; Wardakhan, W.W.; Mohareb, R.M. The Knoevenagel reaction of cyanoacetylhydrazine with pregnenolone: Synthesis of thiophene, thieno[2,3-d]pyrimidine, 1,2,4-triazole, pyran and pyridine derivatives with anti-inflammatory and anti-ulcer activities. *Steroids* **2016**, *107*, 98-111.
44. Katritzky, A.R. *Advances in Heterocyclic Chemistry*, 1st ed., Elsevier S and T: USA; **2011**.
45. Romdhane, A.; Jannet, H.B. Synthesis of new pyran and pyranoquinoline derivatives. *Arab. J. Chem.* **2017**, *10*, S3128-S3134.
46. Calleja, M.C.; Persoone, G. Cyst-based toxicity tests IV: The potential ecotoxicological tests for the prediction of acute toxicity in man as evaluated on the first chemicals of the MEIC. *Alternatives to Laboratory Animals (Atla)* **1992**, *20*, 396-405.
47. Carballo, J.L.; Indal, Z.L.H.; Pérez, P.; Grávalos, M.D.G. A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC Biotech.* **2002**, *2*, 1-5.
48. Chatterjee, P.N.; Roy, S., Alkylation of 1,3-dicarbonyl compounds with benzylic and propargylic alcohols using Ir–Sn bimetallic catalyst: synthesis of fully decorated furans and pyrroles. *Tetrahedron* **2011**, *67*, 4569-4577.
49. Choudhary, M. I.; Thomsen, W.J. *Bioassay Techniques for Drug Development*, Harwood Academic Publishers: U.K.; **2001**; pp. 9-10.
50. Humphrey, P.A.; Zhu, X.; Zarnegar, R.; Swanson P.E.; Ratliff, T.L.; Vollmer, R.T.; Day, M.L. Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. *Am. J. Pathol.* **1995**, *147*, 386-396.
51. Bain, J.; Plater, L.; Elliott, M.; Shpiro, N.; Hastie, J.; McLauchlan, H.; Klervernic, I.; Arthur, S.C.; Alessi, D.R.; Cohen, P. The selectivity of protein kinase inhibitors: A further update. *Biochem. J.* **2007**, *408*, 297-315.
52. Fathi, A.T.; Arowojolu, O.; Swinnen, I.; Sato, T.; Rajkhowa, T.; Small, D.; Marmsater, F.; Robinson, J.E.; Gross, S.D.; Martinson, M.; Alle, S.; Kallan, N.C.; Levis, M. A potential therapeutic target for FLT3-ITD AML: PIM1 Kinase. *Leuk Res.* **2012**, *36*, 224-231.
53. Guo, S.; Mao, X.; Chen, J.; Huang, B.; Jin, C.; Xu, Z.; Qiu, S. Overexpression of Pim-1 in bladder cancer. *J. Exp. Clin. Cancer Res.* **2010**, *29*, 1-7.
54. Wang, J.; Anderson, P.D.; Luo, W.; Gius, D.; Roh, M.; Abdulkadir, S.A. Pim1 kinase is required to maintain tumorigenicity in MYC-expressing prostate cancer cells. *Oncogene.* **2012**, *31*, 1794-1803.
55. Beier, U.H.; Weise, J.B.; Laudien, M.; Sauerwein, H.; Görögh, T. Overexpression of Pim-1 in head and neck squamous cell carcinomas. *Int. J. Oncol.* **2007**, *30*, 1381-1387.
56. Decker, S.; Finter, J.; Forde, A.J.; Kissel, S.; Schwaller, J.; Mack, T.S.; Kuhn, A.; Gray, N.; Follo, M.; Jumaa, H.; Burger, M.; Zirlik, K.; Pfeifer, D.; Miduturu, C.V.; Eibel, H.; Veelken, H.; Dierks, C. PIM Kinases are essential for chronic lymphocytic leukemia cell survival (PIM2/3) and CXCR4-mediated microenvironmental interactions (PIM1). *Mol. Cancer Ther.* **2014**, *13*, 1231-1245.
57. Lu, J.; Zavorotinskaya, T.; Dai, Y.; Niu, X.H.; Castillo, J.; Sim, J.; Yu, J.; Wang, Y.; Langowski, J.L.; Holash, J.; Shannon, K.; Garcia, P.D. Pim2 is required for maintaining multiple myeloma cell growth through modulating TSC2 phosphorylation. *Blood* **2013**, *122*, 1610-1620.
58. Abad, C.C.; Pisonero, H.; Aparicio, B.C.; Roncador, G.; Menchén, A.G.; Climent, J.A., Mata, E.; Rodríguez, M.E.; González, G.M.; Beato, M.S.; Leal, J.F.; Bischoff, J.R.; Piris, M.A. PIM2 inhibition as a rational therapeutic approach in B-cell lymphoma. *Blood* **2011**, *118*, 5517-5527.
59. Chen, L.S.; Redkar, S.; Bearss, D.; Wierda, W.G.; Gandhi, V. Pim kinase inhibitor, SGI-1776, induces apoptosis in chronic lymphocytic leukemia cells. *Blood*, **2009**, *114*, 4150-4157.