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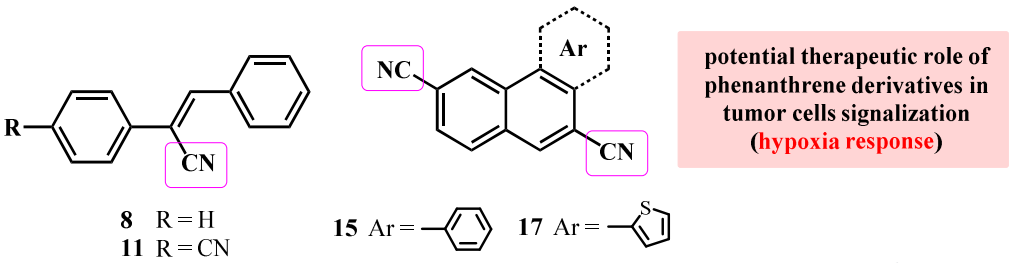
Graphical Abstract



potential therapeutic role of phenanthrene derivatives in tumor cells signalization (hypoxia response)

strong antiproliferative activity and selectivity against HeLa (IC_{50} = 0.21 - 0.65 µM)

Antiproliferative activity and mode of action analysis of novel amino and amido substituted phenantrene and naphtho[2,1-*b*]thiophene derivatives

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Abstract

Herein we present and describe the design and synthesis of novel phenantrene derivatives substituted with either amino or amido side chains and their biological activity. Antiproliferative activities were assessed *in vitro* on a panel of human cancer cell lines. Tested compounds showed moderate activity against cancer cells in comparison with 5-fluorouracile. Among all tested compounds, some compounds substituted with cyano groups showed a pronounced and selective activity in the nanomolar range of inhibitory concentrations against HeLa and HepG2. The strongest selective activity against HeLa cells was observed for acrylonitriles **8** and **11** and their cyclic analogues **15** and **17** substituted with two cyano groups with a corresponding IC_{50} = 0.33, 0.21, 0.65 and 0.45 μM, respectively. Compounds **11** showed the most pronounced selectivity being almost non cytotoxic to normal fibroblasts. Additionally, mode of biological action analysis was performed *in silico* and *in vitro* by Western blot analysis of HIF-1-α relative expression for compounds **8** and **11**.

Keywords: amines, amides, antiproliferative activity, phenatrenes, mode of action analysis, naphtho[2,1-*b*]thiophenes

1. Introduction

Natural products may serve as drugs or templates for design of novel molecules and thus, play a crucial role in the drug discovery and development process. This fact is again gaining interest among researchers in recent years. For example, phenanthrene is a polycyclic aromatic naturally occurring ring system of many biologically active compounds that occur in more than 10 plant families.[1] Hence, natural differently substituted phenantrene derivatives were isolated from Combretaceae, Orchidaceae, Dioscoreaceae and Betulaceae families possessing various biological activities including antitumor,[2-4] antibacterial[5] or anti-inflammatory activities.[6] Most natural phenanthrenes occur in the monomeric form consisting of more than 200 compounds substituted mainly with hydroxy and methoxy groups placed at different positions on the tetracyclic skeleton. Pettit *et al.*[7] have isolated and structurally characterized a series of active phenanthrenes present in the African willow tree *Combretum caffrum*. Their antitumor activity was confirmed on murine P388 lymphocytic leukaemia cell (Fig. 1, **I**). Furthermore, *Lusianthridin* (Fig. 1, **II**) and *Denbinobin* which were isolated from *Dendrobium nobile* were found to exert cytotoxic effects both *in vitro* and *in vivo* on several cancer cells.[8] Phenantrenes isolated from *D. thyrsiflorum* which has been used in Chinese ethnomedicine, also showed antitumor activity on several cancer cell.[9] Additionally, derivatives isolated from *Domohinea perrieri* displayed significant activity towards cancer cells with some cell-type selectivity (Fig. 1, **III**). Obtained results revealed that unsubstituted methyl group placed at the C-7 position is very important for the antitumor activity.[10]

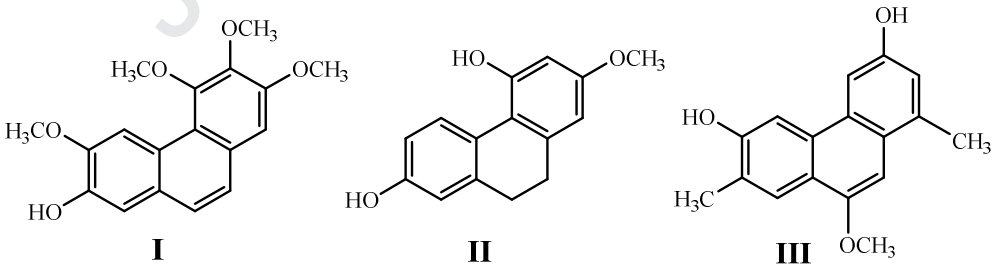


Figure 1. Biologically active phenathrene derivatives

Phenantrene derivatives could be usually prepared by oxidative coupling of the aromatic rings of stilbene precursors. Besides, due to the high biologically potential of substituted phenantrene derivatives and the fact that the synthesis of phenanthrenes is of highly importance in medicinal chemistry, several synthetic methods were published for the

preparation of suchlike compounds. The synthetic strategies for their preparation thus include benzyne-alkyne-benzyne insertion,[11] intramolecular cyclizations,[12] [4+2] benzannulation reactions[13,14] or Pd catalyzed insertion of alkynes into cyclic diaryliodoniums.[15]

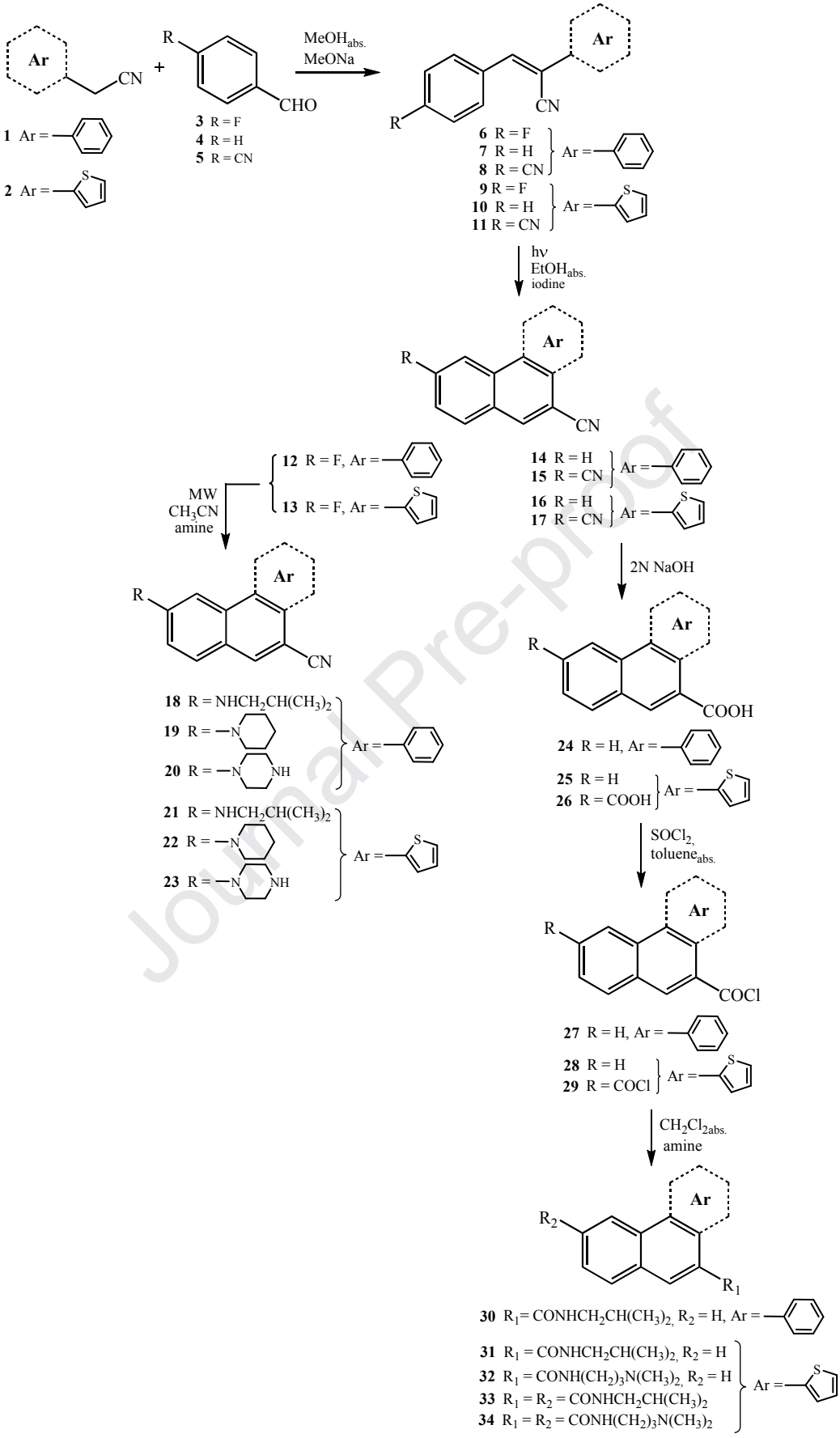
It is well known that heterocyclic derivatives, both those of natural occurrence and widely distributed in nature or those of synthetic origin, are essential for many life processes and have an important role in medicinal chemistry due to the large variety of their possible chemical, pharmacological and biological properties.[16,17] A large number of biologically important compounds belong to a major class of heterocycles containing sulphur. For example, thiophenes and its fused derivatives as benzothiophenes, naphthothiophenes ot thienothiophenes have been classified as highly-privileged structures and valuable building blocks in organic and medicinal chemistry.[18,19] Thiophene nuclei could be found in the structure of numerous medicinal agents including *Raloxifene* as selective estrogen receptor modulator or *Zileuton* for asthma treatment. Naphthothiophene derivatives display a wide range of biological activities like antitumor,[20] antibacterial,[21] antifungal,[22] analgestic[23] or anti-inflammantory[24] and may be additionally exploited as photographic materials[25] or for the purpose of aqueous cold bleaching of textiles.[26] At last, benzothiophenes have been recently proposed also as MAO-B inhibitors with a potential in treatment of neurodegenerative disease as well.[27]

Taking into account that both phenantrene and naphthothiophene derivatives are promising and expanding groups of potentially biologically active compounds whose potential has not yet been thoroughly investigated sufficiently, we have designed and synthesized their novel amino and amido substituted derivatives. As part of the initial evaluation of their biological activity profile**,** their antiproliferative activity was determined. Besides, the structure activity relationships are discussed in this work to correlate between the substituent effects and the activities that aid in drug design. Furthermore, mode of biological action analysis was performed based on obtained results *in silico* and *in vitro*.

2. Results and Discussion

2.1. Chemistry

All newly prepared compounds were synthesized according to the main experimental synthetic procedure presented in Scheme **1.**



Scheme 1. Synthesis of phenantrene and naphtho[2,1-*b*]thiophene derivatives

Acyclic precursors **6–11** were prepared in the reaction of aldol condensation of corresponding benzaldehydes **3–5** and benzylcyanide **1** or 2-(thiophen-2-yl)acetonitrile **2** in absolute methanol using sodium methoxide as a base. Cyclic derivatives of phenanthrene **12–14** and naphtho[2,1-*b*]thiophene **15–17** were prepared by photochemical dehydrocyclization in ethanolic solution with the addition of small amount of iodine, using 400 W high-pressure mercury lamp and Pyrex filter for 2–37 hours. The photochemical dehydrocyclization reaction was monitored by UV/Vis spectroscopy. Targeted amino substituted phenanthrenes **18–20** and naphtho[2,1-*b*]thiophenes **21–23** were prepared from main precursors, namely 3-fluorophenanthrene-9-carbonitrile **12** or 8-fluoronaphtho[2,1-*b*]thiophene-4-carbonitrile **15** by uncatalyzed microwave assisted amination with an excess of added corresponding amine. The reaction was conducted in acetonitrile at 170 °C. Within the aqueous basic hydrolysis of cyano substituted derivatives **14–15** and **16–17** in 2N NaOH, the corresponding carboxylic acids **24–26** were obtained. Carboxylic acids gave in the reaction with thionyl-chloride corresponding acyl-halogenides **27–29** as the main precursors for the synthesis of designed amides. Amide substituted derivatives **30–34** were obtained by reaction of acyl-halogenides **27–29** and an excess amount of the corresponding amine in absolute dichloromethane.

The structures of all prepared compounds were determined by NMR spectroscopy (^{1}H and ^{13}C) based on the analysis of H-H coupling constants as well as chemical shifts and by elemental analysis. Cyclization of acyclic precursors **6–11** into phenantrene and naphtho[2,1-*b*]thiophene derivatives resulted in the disappearance of signals for two H protons confirming thus the formation of tricyclic skeleton. The NMR spectra of carboxylic acids **24-26** showed one additional signal in comparison to spectra of cyano substituted derivatives **14** and **16**–**17**. Introduction of amino substituents into the structure of derivatives **18–23** has been confirmed by appearance of the signals related to protons of amino side chains in the aliphatic part in both ^{1}H and ^{13}C NMR spectra. Additionally, the formation of amide derivatives was confirmed by signal related to the proton of the amide group in the ^{1}H NMR spectra as well as signals related to the protons of amide side chains in aliphatic part of both spectra.

2.2. Antiproliferative activity *in vitro*

Antiproliferative activities of acrylonitrile **6-11,** phenantrene **12-15, 18-20** and **30** and naphtho[2,1-*b*]thiophene derivatives **16-17, 21-23** and **31-34** were assessed on five human tumor cells *in vitro*; HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), HepG2 (hepatocellular carcinoma), CFPAC-1 (ductal pancreatic adenocarcinoma)

and A549 (lung adenocarcinoma) as well as on normal skin fibroblasts HFF. 5-Fluorouracil was used as a standard drug.

Table 1. *In vitro* antiproliferative activity ([a]IC_{50}) of tested derivatives**.**

Comp.	IC_{50}[a] (μM)					
	A549	**CFPAC-1**	**HeLa**	**HepG2**	**SW620**	**HFF**
6	45.76	46.92	3.44	7.73	38.45	52.70
7	64.60	88.19	48.88	81.51	>100	93.73
8	8.39	6.70	0.33	0.52	9.18	6.07
9	40.84	34.24	3.59	5.22	36.41	86.89
10	58.95	97.84	19.99	43.43	>100	84.33
11	47.70	52.35	0.21	0.30	61.39	>100
12	9.57	13.22	8.31	18.33	7.80	3.43
13	49.91	50.98	11.63	32.11	42.42	16.35
14	72.37	80.05	30.31	48.75	>100	>100
15	3.30	8.20	0.65	10.85	6.80	1.78
16	50.43	55.65	29.93	54.28	70.31	84.61
17	5.53	8.19	0.45	7.22	6.04	5.13
18	31.10	35.46	23.63	33.56	43.36	52.91
19	58.91	>100	59.37	81.78	>100	>100
20	5.49	6.19	3.33	19.44	4.62	23.82
21	31.01	18.57	18.56	47.63	41.46	98.42
22	61.30	25.72	32.22	62.01	64.14	62.01
23	7.09	3.66	2.38	20.91	2.58	37.46
30	64.23	68.58	28.42	93.35	61.32	>100
31	63.90	97.52	39.59	75.40	>100	96.82
32	20.23	17.46	14.38	23.19	17.67	23.97
33	27.74	>100	10.76	86.08	0.90	0.74
34	5.57	6.59	4.21	16.23	4.40	5.83
5-FU		0.14	8.81	8.9	0.08	-

[a] IC_{50} values are the concentrations that cause 50% inhibition of cancer cell growth (μM).

According to the obtained results presented in the Table 1, it could be concluded that the majority of tested derivatives showed moderate activity at micromolar range of inhibitory concentrations with some selectivity among tested cell lines. The strongest antiproliferative activity was observed for dicyano substituted acrylonitriles **8** and **11** and their cyclic analogues **15** and **17**. Thus, both acyclic derivatives showed the strongest inhibitory effect and selectivity towards HeLa and HepG2 cell lines in the submicromolar range of inhibitory concentrations. Their cyclic analogues, dicyano substituted phenantrene **15** and naphtho[2,1-*b*]thiophene **17** derivatives displayed selective activity against HeLa cells in submicromolar range of IC_{50} concentrations (IC_{50} 0.65 and 0.45 μM, respectively). Also, obtained results revealed that the thiophene nuclei in the structure of acyclic **11** and cyclic **17** derivatives enhanced the antiproliferative activity in comparison to phenyl analogues **8** and **15**. Fluoro substituted acrylonitriles **6** and **9** showed selective activity against HeLa and HepG2 cells in comparison to other rested cells. Introduction of piperazine group on phenantrene skeleton (**20**) caused enhancement of antiproliferative activity compared to other amino substituted derivatives, namely compounds **18** and **19** with similar results regarding the amino substituted naphtho[2,1-*b*]thiophenes **21-23**. Among amido substituted derivatives **30-34**, the most pronounced activity was observed with di-*N*,*N*-dimethylaminopropyl substituted naphtho[2,1-*b*]thiophene **34**. Mono substituted derivative bearing *N*,*N*-dimethylaminopropyl amide side chain showed slightly improvement of activity in comparison to *N*-isobutyl substituted amide derivatives **30**, **31** and **33**.

Regarding the results of non-cancer cell line HFF, obtained results revealed some selective compounds. Thus, compound **11** showed the most significant selectivity in comparison to the activity against HeLa (IC_{50} 0.21 μM) and HepG2 (IC_{50} 0.230 μM) cells being non cytotoxic against normal skin fibroblasts (IC_{50} > 100 μM).

2.3. Mode of action analysis

2.3.1. Target prediction analysis

PIDGIN analysis of all 23 phenanthrene derivatives against all 1651 potential targets, resulted in 23x1651 = 37973 compound-target interactions. By applying the set threshold, 277 compound-target interactions between the 23 phenanthrene derivatives and 112 unique targets were predicted.

Tested derivatives were found to be active on around half of the known targets (6 out of 13 known targets viz., Q99714, P00352, Q16236, O75164, P11509, and Q12809) of the original phenanthrene (Compound ID: CHEMBL46730). As presented in Fig. 2, phenanthrene derivative **14** has the largest number of predicted targets (49 targets), followed by derivatives **20** and **7** (33 targets), which suggests that these derivatives are less selective towards biological targets. On the other hand, three derivatives (**31, 33**, and **34**) out of 23 are found to be inactive with no predicted targets.

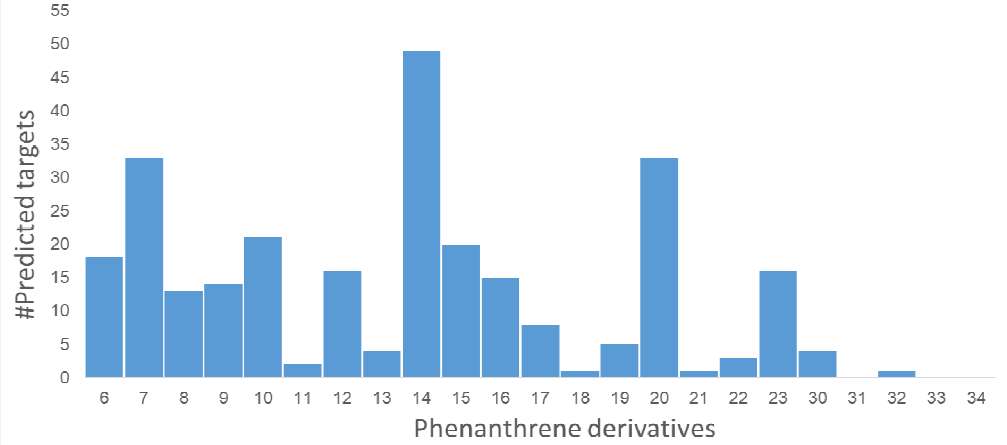


Figure 2. The bar plot displays the number of targets (Y-axis) that is predicted for each derivative (X-axis) when applying the specific threshold ($Pa \geq 0.9$).

As presented in Figure 3, PIDGIN predicts target AR (Androgen receptor, Uniprot ID: P10275) to interact with the largest number of derivatives (10, 43.48% of our input compounds). The prediction of Androgen receptor as a potential target for our Phenanthrene derivative is also supported by literature.[28] AR abnormalities have been identified in various diseases such as androgen insensitivity syndrome, spinal bulbar muscular atrophy, benign prostatic hyperplasia, and prostate cancer.[29] A recent study also demonstrates the association of androgens with depressive symptoms and cognitive status in general populations.[30] Thus, our results open a new insight to explore the therapeutic potential of these derivatives by targeting Androgen receptors to produce anti-cancer and/or anti-depressant activity.

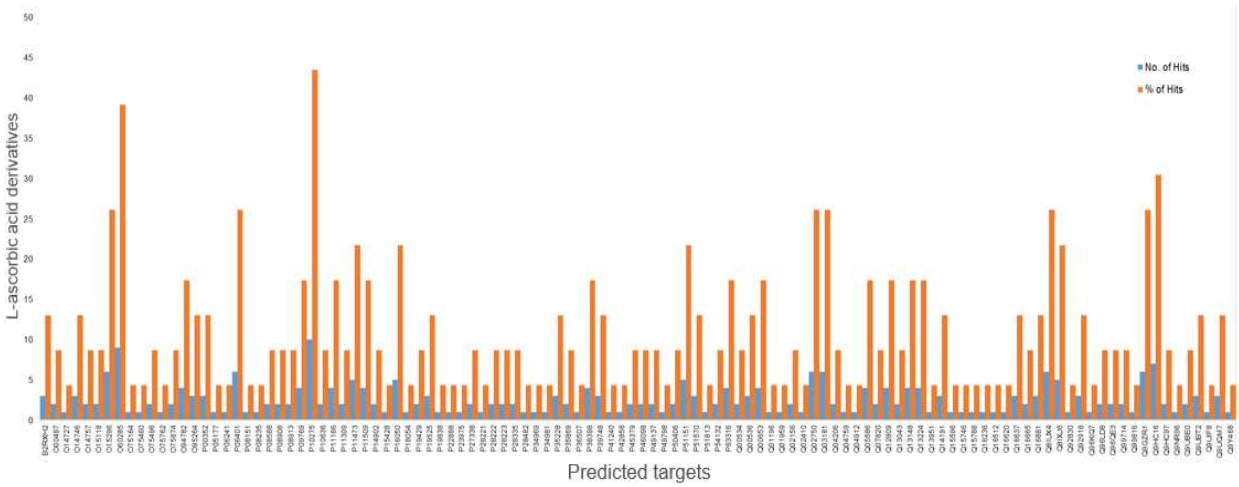


Figure 3. The bar plot showing the number and percentage of Phenathrene derivatives (Y-axis) found to be active for each predicted biological target (X-axis).

To explore the mode of action of Phenanthrene derivatives on various target groups, we have identified the protein classes of our predicted potential targets (Fig. 4).

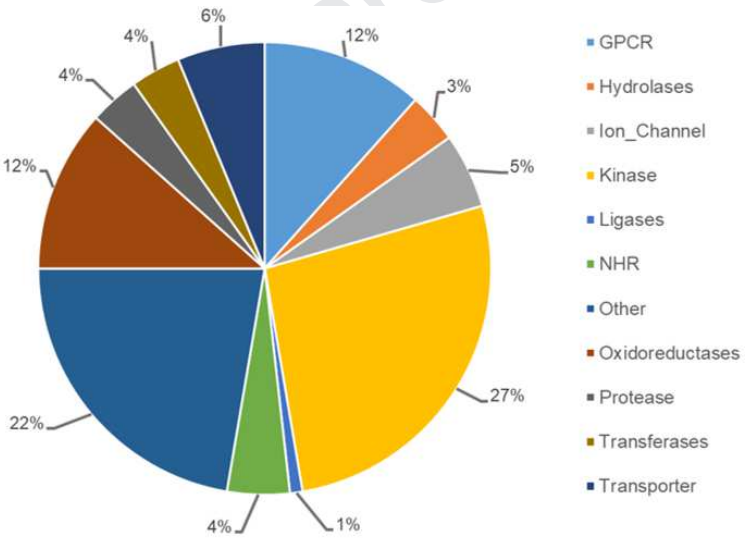


Figure 4. The pie chart shows the percentage distribution of the various target classes for the 112 unique predicted targets of phenanthrene derivatives. The result indicates the presence of high percentage of kinase targets for phenanthrene derivatives compared to other target classes.

The largest proportion of predicted targets belongs to protein kinase target class (27%). Protein kinases play a predominant regulatory role in almost every aspect of cell biology and have ability to modify the function of a protein in almost every conceivable way.[31]

Protein kinases are an important class of drug target for developing therapeutic agents against Cancer, inflammatory diseases, autoimmune disease, neurodegenerative and mental disorders.[32] Interestingly, among the predicted protein kinase targets, NUAK1 (NUAK family SNF1-like kinase 1, Uniprot ID: O60285) and MAP2K1 (Dual specificity mitogen-activated protein kinase kinase 1, Uniprot ID: Q02750), which acts on the largest proportion of phenanthrene derivatives (39.13% and 26.08% respectively), were identified. Both have important role in cancer progression and are potential targets for the treatment of non-small cell lung cancer.[33] In a previous study, Wang et al., has also explored the anti-cancer activity of a phenathrene derivative (T26) by inhibiting a protein kinase.

2.3.2. Functional analysis of the predicted targets

As presented in Figure 5, the functional analysis revealed that predicted targets for phenanthrene derivatives are significantly enriched in seven GO-BP terms, ten GO-MF terms, and five GO-CC terms, which could be categorize into five main terms for both GO-BP & MF and two main terms for GO-CC ontology's.

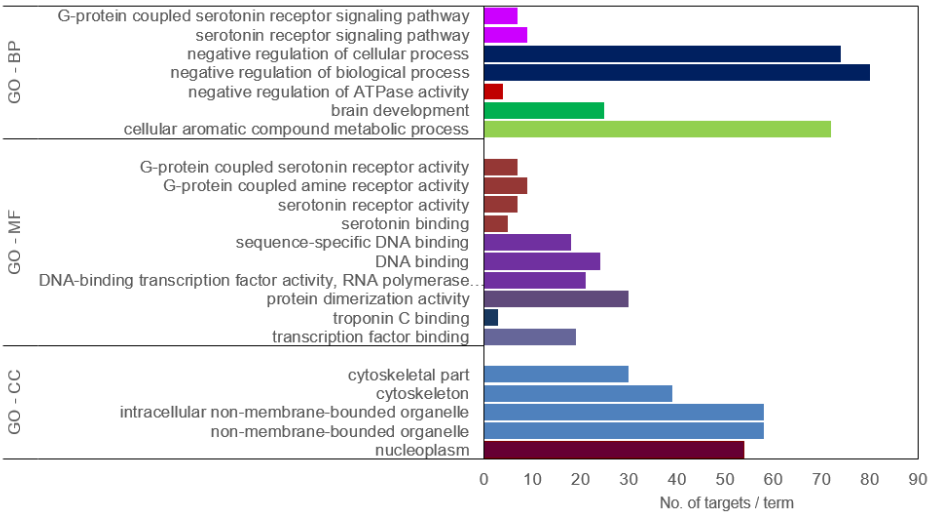


Figure 5. Enriched Gene ontology - Biological Processes (BP), Molecular Function (MF), and Cellular Component (CC) terms for the predicted targets of Phenanthrene derivatives. The vertical axis shows the GO (BP, MF, and CC) terms, and the horizontal axis displays the number of targets per term. The enriched GO terms are presented here with significance level of ≤ 5% after Bonferroni adjustment. The GO terms belonging to same group (same color).

For GO-BP annotation, we find that a large number of the targets are enriched for the negative regulation of biological process term (80 targets) followed by the cellular aromatic compound metabolic process term (72 targets). Significant number of targets is enriched for the serotonin receptor signalling pathway term. For GO-MF annotation, predicted targets of phenanthrene derivatives are significantly enriched with the serotonin receptor activity, DNA binding activity as well as protein dimerization activity. In GO-CC annotation, the predicted targets were found to be enriched with mainly intracellular non-membrane-bounded organelle and nucleoplasm term. The KEGG pathway enrichment analysis was also done and only Serotonergic synapse pathway term was significantly enriched with the predicted targets of tested phenanthrene derivatives (Table 1).

Table 1. Pathway enrichment analysis of the predicted targets of phenanthrene derivatives against the background data set.

Pathway term	No. of targets	% of targets	P- value	Bonferroni (Corrected P-value)	Fold Enrichment
Serotonergic synapse	15	13.39	6.59E-0.5	0.0118	3.31
Acute myeloid leukemia	8	7.14	8.57E-03	0.787	3.32
cAMP signaling pathway	15	13.39	1.73E-02	0.957	1.91
Cocaine addiction	6	5.35	2.30E-02	0.984	3.50
HIF-1 signaling pathway	9	8.03	3.75E-02	0.998	2.26
Prion diseases	4	3.57	4.23E-02	0.999	4.86
Chronic myeloid leukemia	7	6.25	4.84E-02	0.999	2.55

Disease enrichment analysis was also done by using DAVID v6.8 to explore the involvement of the predicted targets of phenanthrene derivatives in various diseases. As presented in Figure 6, predicted targets were significantly enriched in five disease terms, which are mostly related to mental illness. The bar chart showing the significantly enriched disease terms for the predicted targets of phenanthrene derivatives. The vertical axis represents the disease terms, and the horizontal axis represents the number (red color) & percentage (Blue color) of targets per term.

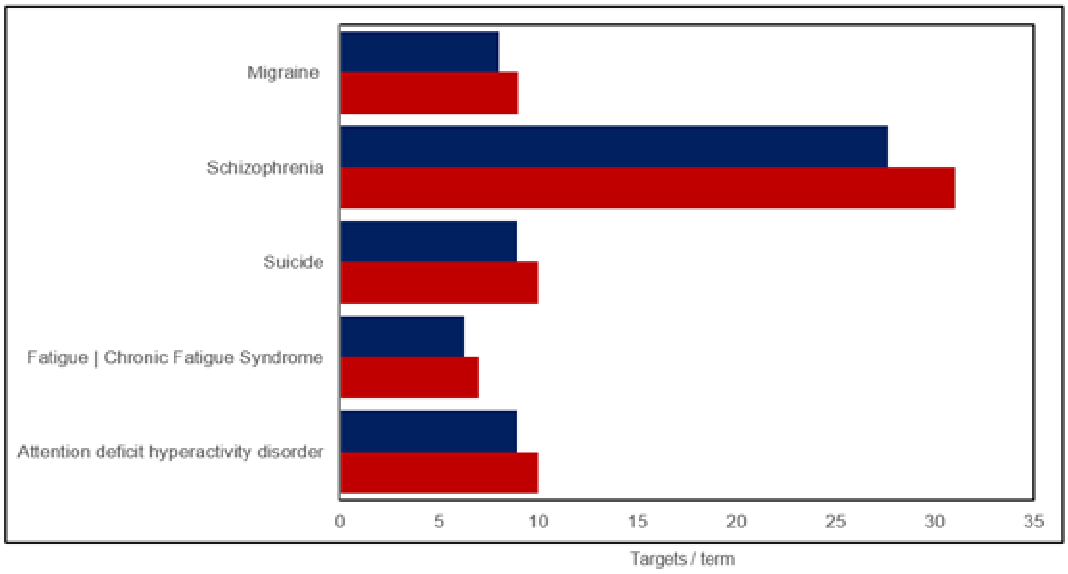


Figure 6. Enriched disease terms for the predicted targets of phenanthrene derivatives.

In summary, we have performed a target prediction analysis of 23 phenanthrene and naphtho[2,1-*b*]thiophene derivatives and predicted a total of 277 compound-target interactions between our derivatives and 112 potential biological targets. Among the predicted targets, the Androgen Receptor (AR) has predicted to interact with large number of phenanthrene and naphtho[2,1-*b*]thiophene derivatives, suggesting anti-cancer and anti-depressant activity. Additionally, the protein kinase target class are found to be the largest target group predicted for our derivatives. Furthermore, the functional enrichment analysis of the predicted targets, suggests the therapeutic role of our phenanthrene and naphtho[2,1-*b*]thiophene derivatives in mental disorders (suggesting Serotonin receptor activity as well as association of predicted targets of our phenanthrene and naphtho[2,1-*b*]thiophene derivatives in mental disorders).

The largest numbers of targets (31, 27.67% of the predicted targets) were enriched for Schizophrenia disease followed by Attention deficit hyperactivity disorder and Suicide disease terms (10, 8.92% of the predicted targets for the both terms) (Fig. 6). In a previous study, Moreno et al., has suggested the role of phenanthrene type alkaloids in the treatment of schizophrenia or Parkinson's disease.[34]

2.4. Western blot analysis of HIF-1-α relative expression

As the *in silico* analysis suggested some of the pathways known to be important in tumor development as potential targets of the tested derivatives, we analysed HIF-1-α relative expression in HeLa and HepG2 cells treated with compounds **8** and **11** due to observed selective antiproliferative effects on these cell lines. The hypoxia response indeed, proved to be an important target for anticancer therapy.[35] Western blot analysis revealed significant up-regulation **(**$p<0.05$) of the hydroxylated HIF-1 protein after treatment of HeLa cells with compounds **8** and **11** after 48h and 72h respectively (Fig. 7). A similar trend was observed in HepG2 cells treated with compounds **8** and **11** but only after 72 h (Fig. 7B). This result is indicative for increased levels of the protein HIF-1α form tagged for proteasomal degradation.[35]

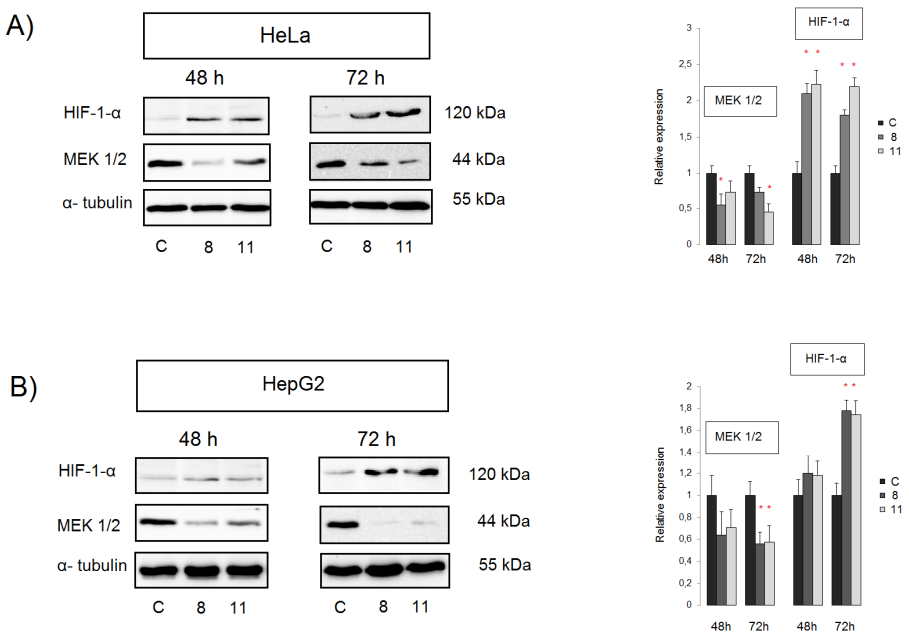


Figure 7. Representative Western blots and summary representation of (A) HIF-1α and (B) MEK 1/2 relative expression levels in HeLa and HepG2 cells treated with compounds **8** and **11** for 48h and 72h. Results are presented as average relative expression values + standard error of the mean (SEM) of chemiluminescent signals obtained in three replicate experiments. Statistically significant changes (Student's t-test, $p<0.05$) are marked with an asterisk (*).

Furthermore, significantly decreased activated MEK1/2 protein levels were observed in HeLa cells treated with compound **8** for 48 h (Fig. 7A) and compound **11** after 72 h-treatment, while in HepG2 cells a significant downregulation of MEK 1/2 was observed only after 72 h treatment with both compounds (Fig. 7B). This result is indicative for deregulation of Ras/Raf/MEK/ERK signaling cascade involved in enhanced dedifferentiation and proliferation of tumor cells (U0126, a mitogen-activated protein kinase kinase 1 and 2 (MEK1 and 2) inhibitor, selectively up-regulates main isoforms of CYP3A subfamily via a pregnane X receptor (PXR) in HepG2 cells; Intrinsically active MEK variants are differentially regulated by proteinases and phosphatases) (Figures 7A and 7B).

3. Conclusion

Herein we described the design and synthesis of novel phenantrene and naphtho[2,1-*b*]thiophene derivatives bearing either different amino or amido side chains. In addition, their antiproliferative activity and mode of biological action are presented and described.

Targeted cyclic derivatives were obtained by using photochemical dehydrocyclization. Amino substituted derivatives were prepared by uncatalyzed microwave assisted amination while amide substituted derivatives obtained by reaction of corresponding acyl-halogenides an excess amount of the amine. The most prominent antiproliferative activity was observed for for dicyano substituted acrylonitriles **8** and **11** and their cyclic analogues **15** and **17**. Additionally both acyclic derivatives showed the selectivity towards HeLa and HepG2 cell lines and were chosen for further structure optimization as a lead compounds for design of more efficient antiproliferative agents. Furthermore, compound **11** did not show cytotoxic effect on normal cells compared to its activity against HeLa and HepG2 cells in nanomolar range of concentrations. The antiproliferative activities were assessed *in vitro* on a several human cancer cell lines as well as on normal skin fibroblasts. Tested compounds showed moderate activity against cancer cells in comparison with 5-fluorouracile. Among the predicted targets *in silico*, the androgen receptor (AR) was predicted to interact with large number of phenanthrene derivatives, suggesting their potential anti-cancer and anti-depressant activity that should be studied separately. Additionally, the protein kinase target class were found to be the largest target group predicted for the new derivatives.

The functional enrichment analysis of the predicted targets, suggests a potential therapeutic role of phenanthrene derivatives in tumor cells signalization, such as for example hypoxia response that was validated in HeLa cells and HepG2 cells.

4. Experimental part

4.1. Synthesis

4.1.1. General methods

All chemicals and solvents were purchased from commercial suppliers Aldrich and Acros. Melting points were recorded on SMP11 Bibby and Büchi 535 apparatus. All NMR spectra were measured in DMSO-d_6 solutions using TMS as an internal standard. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 or Varian Gemini 600 at 300, 600 and 150 and 75 MHz, respectively. Chemical shifts are reported in ppm (δ) relative to TMS. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates.

In preparative photochemical experiments, the irradiation was performed at room temperature with a water-cooled immersion well with "Origin Hanau", 400 W, high-pressure, mercury arc lamp using Pyrex glass as a filter. Elemental analysis for carbon, hydrogen and nitrogen were performed on a Perkin-Elmer 2400 elemental analyzer. Where analyses are indicated only as symbols of elements, analytical results obtained are within 0.4% of the theoretical value.

4.1.2. General method for the synthesis of compounds 6–11

Sodium was dissolved in 10 mL of absolute methanol and benzyl cyanide or 2-(thiophen-2-yl)acetonitrile was added. The mixture was stirred at room temperature for 20 minutes, after that the appropriate aldehyde was added and heated at reflux. The cooled reaction mixture was filtered and, if necessary, recrystallized from the appropriate solvent.

(Z)-3-(4-fluorophenyl)-2-phenylacrylonitrile 6

The reaction of benzyl cyanide **1** (0.98 mL, 8.50 mmol) and 4-fluorobenzaldehyde **3** (0.62 mL, 8.50 mmol) after refluxing for 4 hours and recrystallization from ethanol gave 0.71 g (37%) white powder product; Mp = 114–118 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.06 (s, 1H, $H_{arom,}$), 8.04–7.99 (m, 2H, H_{arom}), 7.79–7.74 (m, 2H, H_{arom}), 7.56–7.45 (m, 3H, H_{arom}), 7.44–7.37 (m, 2H, H_{arom}); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 163.0 (d, J = 249.8 Hz), 141.7, 133.6, 131.6 (d, J = 8.8 Hz), 130.3 (d, J = 3.2 Hz), 129.3, 129.2 (2C), 125.7 (2C), 117.8, 116.1 (d, J = 21.9 Hz), 110.0; Found: C, 80.52; H, 4.70; N, 6.35. Calc. for $C_{15}H_{10}FN$: C, 80.70; H, 4.52; N, 6.27%.

(Z)-2,3-diphenylacrylonitrile 7

The reaction of benzyl cyanide **1** (0.98 mL, 8.50 mmol) and benzaldehyde **4** (0.86 mL, 8.50 mmol) after refluxing for 2 hours and recrystallization from ethanol gave 1.69 g (97%) of white crystalline product; Mp = 88–92 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.06 (s, 1H, $H_{arom.}$), 7.95 (d, J = 7.7 Hz, 2H, H_{arom}), 7.78 (d, J = 7.1 Hz, 2H, H_{arom}), 7.59–7.50 (m, 5H, H_{arom}), 7.48–7.43 (m, 1H, H_{arom}); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 142.9, 133.7, 130.6, 129.3 (2C), 129.2 (2C), 129.1 (2C), 128.9 (2C), 125.8, 117.9, 110.3; Found: C, 87.97; H, 5.20; N, 6.82. Calc. for $C_{15}H_{11}N$: C, 87.77; H, 5.40; N, 6.82%.

(Z)-4-(2-cyano-2-phenylvinyl)benzonitrile 8

The reaction of benzyl cyanide **1** (0.98 mL, 8.50 mmol) and 4-cyanobenzaldehyde **5** (1.12 g, 8.50 mmol) after refluxing for 1.5 hours and recrystallization from ethanol gave 1.89 g (96%) of white powder product; Mp = 174–178 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.16 (s, 1H, $H_{arom.}$), 8.08 (d, J = 8.4 Hz, 2H, H_{arom}), 8.02 (d, J = 8.6 Hz, 2H, H_{arom}), 7.81 (dd, J_1 = 8.2 Hz, J_2 = 1.4 Hz, 2H, H_{arom}), 7.59–7.46 (m, 3H, H_{arom}); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 140.9, 138.1, 133.1, 132.8 (2C), 129.9, 129.6 (2C), 129.3 (2C), 126.1 (2C), 118.4, 117.3, 113.5, 112.3; Found: C, 83.26; H, 4.50; N, 12.24. Calc. for $C_{16}H_{10}N_2$: C, 83.46; H, 4.38; N, 12.17%.

(E)-3-(4-fluorophenyl)-2-(thiophen-2-yl)acrylonitrile 9

The reaction of thiophene-2-acetonitrile **2** (0.86 mL, 8.10 mmol) and 4-fluorobenzaldehyde **3** (0.87 mL, 8.10 mmol) after refluxing for 4 hours and recrystallization from methanol gave 1.51 g, 97%) of a yellow powder product; Mp = 80–86 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 7.98 (dd, J_1 = 8.7 Hz, J_2 = 5.6 Hz, 2H, H_{arom}), 7.82 (s, 1H, H_{arom}), 7.70 (dd, J_1 = 5.0 Hz, J_2 = 0.9 Hz, 1H, H_{arom}), 7.45 (dd, J_1 = 3.6 Hz, J_2 = 0.9 Hz, 1H, H_{arom}), 7.39 (t, J = 8.9 Hz, 2H, H_{arom}), 7.18 (dd, J_1 = 5.0 Hz, J_2 = 3.7 Hz, 1H, H_{arom}); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 163.8 (d, J = 248.5 Hz), 139.0, 138.2, 131.4 (d, J = 8.7 Hz), 129.9 (d, J = 3.2 Hz), 128.4 (2C), 127.8, 126.7 (2C), 116.8, 116.2 (d, J = 21.9 Hz), 104.6; Found: C, 68.25; H, 3.43; N, 6.20. Calc. for $C_{13}H_8FNS$: C, 68.10; H, 3.52; N, 6.11%.

(E)-3-phenyl-2-(thiophen-2-yl)acrylonitrile 10

The reaction of thiophene-2-acetonitrile **2** (0.86 mL, 8.10 mmol) and benzaldehyde **3** (0.83 mL, 8.10 mmol) after refluxing for 4 hours and recrystallization from methanol gave 0.79 g (46%) of a yellow powder product; Mp = 79–83 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 7.91 (d, J = 8.0 Hz, 2H, H_{arom}), 7.82 (s, 1H, H_{arom}), 7.70 (dd, J_1 = 5.1 Hz, J_2 = 1.0 Hz, 1H), 7.57–7.48 (m, 3H, H_{arom}), 7.47 (dd, J_1 = 3.6 Hz, J_2 = 1.0 Hz, 1H, H_{arom}), 7.19 (dd, J_1 = 5.0 Hz, J_2 = 3.7 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 140.7, 138.8, 133.8, 131.1, 129.5 (2C), 129.4 (2C), 128.9, 128.4, 127.3, 117.4, 105.4; Found: C, 74.00; H, 4.10; N, 6.70. Calc. for $C_{13}H_9NS$: C, 73.90; H, 4.29; N, 6.63%.

(E)-4-(2-cyano-2-(thiophen-2-yl)vinyl)benzonitrile 11

The reaction of thiophene-2-acetonitrile **2** (0.86 mL, 8.10 mmol) and 4-cyanobenzaldehyde **5** (1.06 g, 8.10 mmol) after refluxing for 4 hours and recrystallization from methanol gave 1.04 g, 53%) of a light green crystalline product; Mp = 162–165 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.05 (d, J = 8.5 Hz, 2H, H_{arom}), 7.99 (d, J = 8.6 Hz, 2H, H_{arom}), 7.91 (s, 1H, H_{arom}), 7.77 (dd, J_1 = 5.0 Hz, J_2 = 0.9 Hz, 1H, H_{arom}), 7.53 (dd, J_1 = 3.6 Hz, J_2 = 0.8 Hz, 1H, H_{arom}), 7.21 (dd, J_1 = 5.0 Hz, J_2 = 3.8 Hz, 1H, H_{arom}); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 137.8, 137.8, 137.7, 132.7 (2C), 129.4 (2C), 129.0, 128.6, 127.9, 118.4, 116.3, 112.2, 107.9; Found: C, 71.02; H, 3.50; N, 11.60. Calc. for $C_{14}H_8N_2S$: C, 71.16; H, 3.41; N, 11.86%.

4.1.3. General method for the synthesis of compounds 12 - 17

Ethanolic solutions of acrylonitriles **6 – 11** and small amount of iodine (5%) were irradiated at room temperature with 400 W, high-pressure mercury lamp using a Pyrex filter for 2–37 hours, until the UV spectra showed that the reaction of photochemical dehydrocyclization was completed. The air was bubbled through the solution. The solutions were concentrated under reduced pressure and resulting product was filtered off.

3-fluorophenanthrene-9-carbonitrile 12

Compound **12** was prepared from **6** 0.30 g (1.30 mmol) in ethanol (400 mL) after irradiation for 4 hours to yield 0.14 (47%) of light yellow powder. Mp = 159–170 °C

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.96 (dd, J_1 = 7.1 Hz, J_2 = 2.1 Hz, 1H, H_{arom}), 8.76 (dd, J_1 = 11.4 Hz, J_2 = 2.3 Hz, 1H, H_{arom}), 8.71 (s, 1H, H_{arom}), 8.25 (dd, J_1 = 8.9 Hz, J_2 = 6.1 Hz, 1H, H_{arom}), 8.18 (dd, J_1 = 6.9 Hz, J_2 =2.3 Hz, 1H, H_{arom}), 7.95–7.83 (m, 2H, H_{arom}), 7.70 (td, J_1 = 8.6 Hz, J_2 = 2.5 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 163.6 (d, J = 248.4 Hz), 136.2, 133.8 (d, J = 9.5 Hz), 133.1 (d, J = 9.7 Hz), 129.7, 129.6 (d, J = 4.2 Hz), 128.9, 128.7, 126.9, 125.6, 124.9, 118.0, 117.6 (d, J = 24.3 Hz), 109.3 (d, J = 23.2 Hz), 107.8; Found: C, 81.14; H, 3.84; N, 6.39. Calc. for $C_{15}H_8FN$: C, 81.44; H, 3.65; N, 6.33%.

8-fluoronaphtho[2,1-b]thiophene-4-carbonitrile 13

Compound **13** was prepared from **9** 0.30 g (1.31 mmol) in ethanol (400 mL) after irradiation for 26 hours to yield 0.12 (37%) of brown powde; Mp = 170–176 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.64 (s, 1H, H_{arom}), 8.47 (dd, J_1 = 10.51 Hz, J_2 = 2.63 Hz, 1H, H_{arom}), 8.38 (d, J = 5.42 Hz, 1H, H_{arom}), 8.27 (dd, J_1 = 9.01 Hz, J_2 = 5.93 Hz, 1H, H_{arom}), 8.16 (d, J = 5.42 Hz, 1H, H_{arom}), 7.63 (td, J_1 = 8.83 Hz, J_2 = 2.64 Hz, 1H, H_{arom}); ^{13}C NMR (151 MHz, DMSO) (δ/ppm): 163.4 (d, J = 247.8 Hz), 136.4 (d, J = 6,5 Hz), 132.6 (d, J = 9,87 Hz), 132.2, 131.7 (d, J = 10,3 Hz), 128.9, 126.6, 123.8, 117.2, 116. (d, J = 24,8 Hz), 108.9 (d, J = 22,3 Hz), 103.0;

Found: C, 68.51; H, 2.75; N, 6.20. Calc. for $C_{13}H_6FNS$: C, 68.71; H, 2.66; N, 6.16%.

Phenanthrene-9-carbonitrile 14

Compound **14** was prepared from **7** 0.30 g (1.50 mmol) in ethanol (400 mL) after irradiation for 2.5 hours to yield 0.12 (41%) of orange powder; Mp = 110–116 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 9.02–8.96 (m, 1H, H_{arom}), 8.94 (d, J = 8.4 Hz, 1H, H_{arom}), 8.70 (s, 1H, H_{arom}), 8.23–8.13 (m, 2H, H_{arom}), 7.95–7.85 (m, 3H, H_{arom}), 7.80 (t, J = 7.5 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 136.7, 131.7, 130.8, 130.2, 130.0, 129.9, 129.1, 129.0, 128.5, 128.4, 125.6, 124.3, 123.7, 118.1, 108.5; Found: C, 88.76; H, 4.35; N, 6.89. Calc. for $C_{15}H_9N$: C, 88.64; H, 4.46; N, 6.89%.

Phenanthrene-3,9-dicarbonitrile 15

Compound **15** was prepared from **8** 0.50 g (1.90 mmol) in ethanol (400 mL) after irradiation for 4 hours to yield 0.30 (69%) of yellow crystals; Mp = 279–284 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 9.52 (s, 1H, H_{arom}), 9.13–9.06 (m, 1H, H_{arom}), 8.77 (s, 1H, H_{arom}), 8.31 (d, J = 8.3 Hz, 1H, H_{arom}), 8.25–8.18 (m, 1H, H_{arom}), 8.12 (d, J = 8.3 Hz, 1H, H_{arom}), 7.99–7.90 (m, 2H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 135.7, 132.6, 132.1, 131.4, 131.3, 130.8, 130.5, 130.0, 129.7, 129.4, 129.4, 128.8, 128.5, 128.0, 125.8, 124.8, 119.2, 117.5, 112.9, 111.7; Found: C, 83.89; H, 3.75; N, 12.36. Calc. for $C_{16}H_8N_2$: C, 84.17; H, 3.53; N, 12.27%.

Naphtho[2,1-b]thiophene-4-carbonitrile 16

Compound **16** was prepared from **10** 0.50 g (2.37 mmol) in ethanol (800 mL) after irradiation for 9 hours to yield 0.12 (23%) of brown powder; Mp = 126–132 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.64–8.62 (m, 2H, H_{arom}), 8.39 (d, J = 5.4 Hz, 1H, H_{arom}), 8.18 (d, J = 8.9 Hz, 1H, H_{arom}), 8.16 (d, J = 5.5 Hz, 1H, H_{arom}), 7.87 (t, J = 7.1 Hz, 1H,

H_{arom}), 7.73 (t, *J* = 7.6 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (*δ*/ppm): 137.2, 135.9, 132.9, 130.6, 130.5, 129.9, 129.9, 129.4, 127.4, 124.7, 123.9, 117.8, 104.1; Found: C, 74.35; H, 3.21; N, 6.60. Calc. for $C_{13}H_7NS$: C, 74.16; H, 3.37; N, 6.69%.

Naphtho[2,1-b]thiophene-4,8-dicarbonitrile 17

Compound **17** was prepared from **11** 0.30 g (1.27 mmol) in ethanol (400 mL) after irradiation for 2 hours to yield 0.26 (90%) of pink crystals; Mp = >300 °C.

^{1}H NMR (300 MHz, DMSO) (*δ*/ppm): 9.30 (s, 1H, H_{arom}), 8.72 (s, 1H, H_{arom}), 8.51 (s, 1H, H_{arom}), 8.39–8.23 (m, 2H, H_{arom}), 8.02 (d, *J* = 7.0 Hz, 1H, H_{arom});

^{13}C NMR (150 MHz, DMSO) (*δ*/ppm): 136.8, 131.8, 131.2, 130.7, 130.5, 130.3, 129.2, 127.7, 123.8, 118.7, 116.8, 111.9, 106.6; Found: C, 71.65; H, 2.65; N, 12.01. Calc. for $C_{14}H_6N_2S$: C, 71.78; H, 2.58; N, 11.96%.

4.1.4. General method for preparation of compounds 18–23

Compounds **18–23** were prepared using microwave irradiation, at optimized reaction time with power 800 W and 40 bar pressure, from compound **12** or **15** in acetonitrile (10 mL) with excess of added corresponding amine. After cooling, the resulting product was separated by column chromatography on SiO_2 using dichloromethane/methanol as eluent.

3-(N-isobutylamino)phenanthrene-9-carbonitrile 18

Compound **18** was prepared using above described method from **12** (0.10 g, 0.46 mmol) and *i*-butylamine (1.50 mL, 15.80 mmol) after 29 hours of irradiation to yield 0.06 g (45%) of brown oil.

^{1}H NMR (300 MHz, DMSO) (*δ*/ppm): 8.76 (d, *J* = 7.4 Hz, 1H, H_{arom}), 8.34 (s, 1H, H_{arom}), 8.03 (d, *J* = 7.2 Hz, 1H, H_{arom}), 7.79 (d, *J* = 8.8 Hz, 1H, H_{arom}), 7.76–7.69 (m, 2H, H_{arom}), 7.66 (d, *J* = 1.5 Hz, 1H, H_{arom}), 7.14 (dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz, 1H, H_{arom}), 6.72 (t, *J* = 5.5 Hz, 1H, NH), 3.12 (t, *J* = 6.6 Hz, 2H, CH_2), 1.96 (p, *J* = 6.7 Hz, 1H, CH), 1.02 (d, *J* = 6.6 Hz, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO) (*δ*/ppm): 151.6, 136.5, 134.3, 131.3, 129.6, 128.9, 128.5, 127.5, 125.2, 124.3, 121.3, 119.3, 117.4, 101.0, 100.3, 50.7, 27.9, 20.9 (2C); Found: C, 83.01; H, 6.74; N, 10.25. Calc. for $C_{19}H_{18}N_2$: C, 83.18; H, 6.61; N, 10.21%.

3-(N-piperidinyl)phenanthrene-9-carbonitrile 19

Compound **19** was prepared using above described method from **12** (0.20 g, 0.92 mmol) and piperidine (1.80 mL, 17.39 mmol) after 14 hours of irradiation to yield 0.07 g (8%) of brown oil.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.94 (d, J_1 = 7.5 Hz, 1H, H_{arom}), 8.43 (s, 1H, H_{arom}), 8.10–8.04 (m, 2H, H_{arom}), 7.92 (d, J = 9.0 Hz, 1H, H_{arom}), 7.82–7.70 (m, 2H, H_{arom}), 7.49 (dd, J_1 = 9.0 Hz, J_2 = 2.2 Hz, 1H, H_{arom}), 3.54 (s, 4H, CH_2), 1.66 (s, 6H, CH_2); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 152.7, 136.3, 133.6, 131.3, 129.3, 128.7, 127.8, 125.3, 124.6, 122.2, 119.0, 117.9, 105.2, 102.9, 48.9 (2C), 25.5 (2C), 24.5; Found: C, 83.65; H, 6.50; N, 9.85. Calc. for $C_{20}H_{18}N_2$: C, 83.88; H, 6.34; N, 9.78%.

3-(N-piperazinyl)phenanthrene-9-carbonitrile 20

Compound **20** was prepared using above described method from **12** (0.10 g, 0.46 mmol) and piperazine (0.56 g, 7.28 mmol) after 14 hours of irradiation to yield 0.07 g (48%) of orange oil. ^{1}H NMR (600 MHz, DMSO) (δ/ppm): 8.97 (d, J = 8.0 Hz, 1H, H_{arom}), 8.47 (s, 1H, H_{arom}), 8.11 (s, 1H, H_{arom}), 7.96 (d, J = 9.0 Hz, 1H, H_{arom}), 7.83–7,74 (m, 2H, H_{arom}), 7.55–7.49 (m, 1H, H_{arom}), 3.54 (s, 4H, CH_2), 3.04 (s, 4H, CH_2); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 152.5, 136.3, 133.5, 131.3, 129.4, 128.8, 127.9, 125.3, 124.7, 122.8, 118.9, 117.9, 105.7, 103.6, 47.5 (2C), 44.9 (2C); Found: C, 79.71; H, 5.90; N, 14.38. Calc. for $C_{19}H_{17}N_3$: C, 79.41; H, 5.96; N, 14.62%.

8-(N-isobutylamino)naphtho[2,1-b]thiophene-4-carbonitrile 21

Compound **21** was prepared using above described method from **15** (0.10 g, 0.48 mmol) and *i*-butylamine (1.18 mL, 11.95 mmol) after 37 hours of irradiation to yield 0.04 g (36%) of light brown powder. Mp = 136–138 °C

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.23 (s, 1H, H_{arom}), 8.18 (d, J = 5.4 Hz, 1H, H_{arom}), 7.94 (d, J = 5.4 Hz, 1H, H_{arom}), 7.79 (d, J = 8.9 Hz, 1H, H_{arom}), 7.28 (d, J = 1.8 Hz, 1H, H_{arom}), 7.11 (dd, J_1 = 8.9, J_2 = 2.1 Hz, 1H, H_{arom}), 6.72 (t, J = 5.5 Hz, 1H, NH), 3.12–3.04 (t, J = 5.9 Hz, 2H, CH_2), 1.99–1.90 (m, 1H, CH), 1.01 (d, J = 6.6 Hz, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 151.2, 135.1, 133.5, 132.7, 130.8, 126.9, 124.0, 122.0, 118.9, 117.9, 99.6, 96.9, 50.7, 27.9, 20.9 (2C); Found: C, 72.96; H, 5.60; N, 9.90. Calc. for $C_{17}H_{16}N_2S$: C, 72.82; H, 5.75; N, 9.99%.

8-(N-piperidinyl)naphtho[2,1-b]thiophene-4-carbonitrile 22

Compound **22** was prepared using above described method from **15** (0.10 g, 0.48 mmol) and piperidine (0.94 mL, 9.56 mmol) after 31 hours of irradiation to yield 0.05 g (41%) of yellow powder; Mp = 147–149 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.34 (d, J = 5,6 Hz, 1H, H_{arom}), 8.32 (s, 1H, H_{arom}), 7.98 (d, J = 5.4 Hz, 1H, H_{arom}), 7.93 (d, J = 9.2 Hz, 1H, H_{arom}), 7.72 (d, J = 2.2 Hz, 1H, H_{arom}),

7.46 (dd, J_1 = 9.2 Hz, J_2 = 2.4 Hz, 1H, H_{arom}), 3.51 (bs, 4H, CH_2), 1.65 (bs, 6H, CH_2); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 152.3, 135.8, 132.8, 132.5, 130.9, 127.4, 124.3, 122.8, 118.7, 117.8, 105.1, 104.7, 98.7, 48.8 (2C), 25.5 (2C), 24.5; Found: C, 73.77; H, 5.38; N, 9.73. Calc. for $C_{18}H_{16}N_2S$: C, 73.94; H, 5.52; N, 9.58%.

8-(N-piperazinyl)naphtho[2,1-b]thiophene-4-carbonitrile 23

Compound **23** was prepared using above described method from **15** (0.10 g, 0.48 mmol) and piperazine (0.58 mg, 6.69 mmol) after 30 hours of irradiation to yield 0.06 g (23%) of light brown powder; Mp = 147–149 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.41 (s, 1H, H_{arom}), 8.39 (d, J = 5.5 Hz, 1H, H_{arom}), 8.05 (d, J =4.0 Hz, 1H, H_{arom}), 8.03 (d, J = 7.8 Hz, 1H, H_{arom}), 7.86 (d, J = 2.1 Hz, 1H, H_{arom}), 7.53 (dd, J_1 = 9.1 Hz, J_2 = 2.4 Hz, 1H, H_{arom}), 3.79–3.61 (m, 4H, CH_2), 3.33–3.27 (m, 4H, CH_2); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 151.1, 136.6, 136.0, 132.5, 132.4, 131.2, 128.0, 124.2, 123.8, 118.5, 118.0, 106.3, 100.0, 45.3 (2C), 43.2 (2C); Found: C, 69.79; H, 5.01; N, 14.50. Calc. for $C_{17}H_{15}N_3S$: C, 69.60; H, 5.15; N, 14.32%.

4.1.5. General method for the synthesis of compounds 24–26

2 N aqueous solution of sodium hydroxide and compounds **13, 16** and **17** were refluxed for 24 hours. Cooled reaction mixture was poured into ice, and resulting product was filtered off.

Phenanthrene-9-carboxylic acid 24

Compound **24** was prepared using above described method, from phenanthrene-9-carbonitrile **13** (0.20 g, 0.98 mmol) and 2 N aqueaus solution of sodium hydroxide (19.0 mL) to yield 0.15 g (71%) of grey powder; Mp 208–216 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.87 (t, J = 8.8 Hz, 2H, H_{arom}), 8.33 (d, J = 7.7 Hz, 1H, H_{arom}), 8.12 (s, 1H, COOH), 8.06 (d, J = 8.0 Hz, 1H, H_{arom}), 7.99 (s, 1H, H_{arom}), 7.80–7.63 (m, 4H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 171.1, 134.0, 130.6, 130.6, 130.4, 129.5, 128.8, 128.3, 127.7, 127.5, 127.5, 126.8, 126.3, 123.6, 123.3; Found: C, 81.33; H, 4.67. Calc. for $C_{15}H_{10}O_2$: C, 81.07; H, 4.54%.

Naphtho[2,1-b]thiophene-4-carboxylic acid 25

Compound **25** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4-carbonitrile **16** (0.14 g, 0.65 mmol) and 2 N aqueaus solution of sodium hydroxide (13.0 mL) to yield 0.13 g (91%) of grey powder; Mp 283–286 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.56 (d, J = 8.2 Hz, 1H, H_{arom}), 8.51 (s, 1H, H_{arom}), 8.35 (s, 1H, COOH), 8.23 (d, J = 5.6 Hz, 1H, H_{arom}), 8.06 (d, J = 8.1 Hz, 1H, H_{arom}), 7.96 (d, J = 5.5 Hz, 1H, H_{arom}), 7.75 (t, J = 7.6 Hz, 1H, H_{arom}), 7.64 (t, J = 7.0 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): not enouht soluble; Found: C, 68.70; H, 3.26. Calc. for $C_{13}H_8O_2S$: C, 68.40; H, 3.53%.

Naphtho[2,1-b]thiophene-4,8-dicarboxylic acid 26

Compound **26** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4,8-dicarbonitrile **17** (0.30 g, 0.613 mmol) and 2 N aqueaus solution of sodium hydroxide (20.0 mL) to yield 0.30 g (86%) of light yellow powder; Mp >300 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 13.52 (s, 2H, COOH), 9.13 (s, 1H, H_{arom}), 8.69 (s, 1H, H_{arom}), 8.39 (d, J = 5.6 Hz, 1H, H_{arom}), 8.36 (d, J = 8.7 Hz, 1H, H_{arom}), 8.14–8.08 (m, 2H, H_{arom}); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 167.3, 166.9, 137.7, 135.3, 132.3, 131.1, 130.7, 130.4, 129.8, 128.0, 125.7, 125.4, 124.9, 121.8; Found: C, 61.95; H, 2.72. Calc. for $C_{14}H_8O_4S$: C, 61.76; H, 2.96%.

4.1.6. General method for the synthesis of compounds 27–29

A mixture of corresponding carboxylic acids **26–26** and thionyl chloride in absolute toluene was refluxed for 19 hours. Toluene and excess of thionyl chloride was removed under reduced pressure. The crude product was washed 3 times with absolute toluene to obtained powdered product.

Phenanthrene-9-carbonyl chloride 27

Compound **27** was prepared using above described method, from phenanthrene-9-carboxylic acid **24** (0.11 g, 0.51 mmol), absolute toluene (10 mL) and 0.37 mL thionyl chloride to yield 0.11 g (91%) of yellow powder.

^{1}H NMR (600 MHz, DMSO) (δ/ppm): 9.00–8.99 (m, 1H, H_{arom}), 8.95 (d, J = 8.4 Hz, 1H, H_{arom}), 8.89 (d, J = 8.0 Hz, 1H, H_{arom}), 8.72 (s, 1H, H_{arom}), 8.20–8.19 (m, 1H, H_{arom}), 8.17 (d, J = 7.9 Hz, 1H, H_{arom}), 7.92 (t, J = 7.6 Hz, 1H, H_{arom}), 7.80 (t, J = 7.4 Hz, 1H, H_{arom}), 7.76–7.72 (m, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): not enouht soluble; Found: C, 74.70; H, 3.98. Calc. for $C_{15}H_9ClO$: C, 74.85; H, 3.77%.

Naphtho[2,1-b]thiophene-4-carbonyl chloride 28

Compound **28** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4-carboxylic acid **25** (0.10 g, 0.44 mmol), absolute toluene (10 mL) and 0.37 mL thionyl

chloride to yield 0.10 g (96%) of yellow powder.

^{1}H NMR (600 MHz, DMSO) (δ/ppm): 8.63 (s, 1H, H_{arom}), 8.58 (d, J = 8.3 Hz, 1H, H_{arom}), 8.28 (d, J = 5.5 Hz, 1H, H_{arom}), 8.23 (d, J = 8.0 Hz, 1H, H_{arom}), 8.01 (d, J = 5.5 Hz, 1H, H_{arom}), 7.80–7.76 (m, 1H, H_{arom}), 7.66–7.62 (m, 1H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 167.6, 137.5, 131.1, 130.5 (2C), 129.6, 129.2, 126.6, 124.4, 123.5, 122.3, 120.9; Found: C, 63.50; H, 2.75; N, 6.35. Calc. for $C_{13}H_7ClOS$: C, 63.29; H, 2.86%.

Naphtho[2,1-b]thiophene-4,8-dicarbonyl dichloride 29

Compound **29** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4,8-dicarboxylic acid **26** (0.50 g, 1.83 mmol), absolute toluene (10 mL) and 1.32 mL thionyl chloride to yield 0.49 g (86%) of yellow powder.

^{1}H NMR (600 MHz, DMSO) (δ/ppm): 9.13 (s, 1H, H_{arom}), 8.69 (s, 1H, H_{arom}), 8.39 (d, J = 5.5 Hz, 1H, H_{arom}), 8.36 (d, J = 8.5 Hz, 1H, H_{arom}), 8.13–8.08 (m, 2H, H_{arom}); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 167.2, 166.8, 137.7, 135.3, 132.3, 131.1, 130.7, 130.4, 129.8, 128.00, 125.7, 125.4, 124.9, 121.7; Found: C, 54.55; H, 2.06; N, 6.35. Calc. for $C_{14}H_6Cl_2O_2S$: C, 54.39; H, 1.96%.

4.1.7. General method for the synthesis of compounds 30–34

A mixture of carbonyl chlorides **27**–**29** and excess of corresponding amine in dry dichloromethane was stirred at room temperature for 2 hours. The mixture was washed with 10 mL of 20% Na_2CO_3 and 10 mL water. After drying over $MgSO_4$, the organic layer was concentrated at reduced pressure.

N-isobutylphenanthrene-9-carboxamide 30

Compound **30** was prepared using above described method, from phenanthrene-9-carbonyl chloride **27** (0.37 g, 1.52 mmol), dry dichloromethane (20 mL) and 0.91 mL (9.15 mmol) *i*-butylamine to obtain 0.04 g (10%) of white powder. Mp = 177–179 °C

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.88 (t, J = 9.2 Hz, 2H, H_{arom}), 8.68 (t, J = 5.7 Hz, 1H, NH), 8.20 (d, J = 7.8 Hz, 1H, H_{arom}), 8.08 (d, J = 7.6 Hz, 1H, H_{arom}), 7.92 (s, 1H, H_{arom}), 7.79–7.65 (m, 4H, H_{arom}), 3.20 (t, J = 6.4 Hz, 2H, CH_2), 1.96–1.88 (m, 1H, CH), 0.98 (d, J = 6.7 Hz, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 169.1, 134.6, 130.7, 130.5, 130.4, 129.5, 128.9, 128.3, 127.7, 127.5, 127.5, 126.6, 126.1, 123.7, 123.3, 47.0, 28.7, 20.7 (2C); Found: C, 82.36; H, 6.98; N, 4.87. Calc. for $C_{19}H_{19}NO$: C, 82.28; H, 6.90; N, 5.05%.

***N*-isobutylnaphtho[2,1-b]thiophene-4-carboxamide 31**

Compound **31** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4-carbonyl chloride **28** (0.10 g, 0.41 mmol), dry dichloromethane (10 mL) and 0.24 mL (2.43 mmol) *i*-butylamine to obtain 0.06 g (49%) of white powder; Mp = 148–152 °C.

^{1}H NMR (300 MHz, DMSO) (δ/ppm): 8.88 (t, *J* = 5.7 Hz, 1H, NH), 8.56 (d, *J* = 8.2 Hz, 1H, H_{arom}), 8.47 (s, 1H, H_{arom}), 8.24 (d, *J* = 5.6 Hz, 1H, H_{arom}), 8.10 (d, *J* = 7.8 Hz, 1H, H_{arom}), 7.97 (d, *J* = 5.5 Hz, 1H, H_{arom}), 7.75 (t, *J* = 6.9 Hz, 1H, H_{arom}), 7.65 (t, *J* = 8.0 Hz, 1H, H_{arom}), 3.20 (t, *J* = 6.4 Hz, 2H, CH_2), 2.00–1.89 (m, 1H, CH), 0.96 (d, *J* = 6.7 Hz, 6H, CH_3);

^{13}C NMR (75 MHz, DMSO) (δ/ppm): 166.5, 137.3, 135.1, 130.8, 130.5, 130.3, 129.7, 128.6, 127.1, 126.5, 124.4, 124.3, 121.8, 47.3, 28.6, 20.7 (2C); Found: C, 72.30; H, 6.35; N, 4.70. Calc. for $C_{17}H_{17}NOS$: C, 72.05; H, 6.05; N, 4.94%.

***N*-(3-(*N*',*N*'-dimethylamino)propyl)naphtho[2,1-b]thiophene-4-carboxamide 32**

Compound **32** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4-carbonyl chloride **28** (0.14 g, 0.55 mmol), dry dichloromethane (12 mL) and 0.41 mL (3.28 mmol) *N*,*N*-dimethylaminopropyl-1-amine to obtain 0.07 g (41%) of yellow oil.

^{1}H NMR (600 MHz, DMSO) (δ/ppm): 8.95 (t, *J* = 5.4 Hz, 1H, NH), 8.57 (d, *J* = 8.1 Hz, 1H, H_{arom}), 8.44 (s, 1H, H_{arom}), 8.24 (d, *J* = 5.5 Hz, 1H, H_{arom}), 8.09 (d, *J* = 8.0 Hz, 1H, H_{arom}), 7.98 (d, *J* = 5.5 Hz, 1H, H_{arom}), 7.76–7.72 (m, 1H, H_{arom}), 7.66–7.63 (m, 1H, H_{arom}), 3.41 (q, *J* = 6.6 Hz, 2H, CH_2), 2.43 (t, *J* = 7.0 Hz, 2H, CH_2), 2.25 (s, 6H, CH_3), 1.80–1.75 (m, 2H, CH_2);

^{13}C NMR (150 MHz, DMSO) (δ/ppm): 165.9, 136.8, 134.5, 130.4, 130.0, 129.8, 129.2, 128.2, 126.5, 126.0, 123.8, 123.7, 121.3, 56.6, 44.8 (2C), 37.7, 26.8; Found: C, 69.01; H, 6.56; N, 8.25. Calc. for $C_{18}H_{20}N_2OS$: C, 69.20; H, 6.45; N, 8.97%.

***N*^{4},*N*^{8}-diisobutylnaphtho[2,1-b]thiophene-4,8-dicarboxamide 33**

Compound **33** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4,8-dicarbonyl dichloride **29** (0.23 g, 0.72 mmol), dry dichloromethane (20 mL) and 0.43 mL (4.31 mmol) *i*-butylamine to obtain 0.02 g (4%) of white powder; Mp = 187–190 °C.

^{1}H NMR (600 MHz, DMSO) (δ/ppm): 9.03 (s, 1H, H_{arom}), 8.95 (t, *J* = 5.6 Hz, 1H, NH), 8.78 (t, *J* = 5.6 Hz, 1H, NH), 8.49 (s, 1H), 8.29 (d, *J* = 5.6 Hz, 1H, H_{arom}), 8.16 (d, *J* = 8.5 Hz, 1H, H_{arom}), 8.08–8.04 (m, 2H, H_{arom}), 3.21–3.18 (m, 4H, CH_2), 1.94–1.90 (m, 2H, CH), 0.96 (d, *J* = 6.0 Hz, 6H, CH_3), 0.95 (d, *J* = 6.0 Hz, 6H, CH_3); ^{13}C NMR (150 MHz, DMSO) (δ/ppm): 166.0, 165.9, 137.4, 135.0, 133.7, 131.3, 131.0, 129.3, 128.9, 127.9, 124.5, 123.2, 122.7, 121.4, 46.9, 46.8, 28.2, 28.1, 20.3, 20.3; Found: C, 69.19; H, 6.70; N, 7.15. Calc. for

$C_{22}H_{26}N_2O_2S$: C, 69.08; H, 6.85; N, 7.32%.

N^4,N^8-bis(3-(N',N'-dimethylamino)propyl)naphtho[2,1-b]thiophene-4,8-dicarboxamide 34

Compound **34** was prepared using above described method, from naphtho[2,1-*b*]thiophene-4,8-dicarbonyl dichloride **29** (0.23 g, 0.72 mmol), dry dichloromethane (20 mL) and 0.54 mL (4.31 mmol) 3-*N*,*N*-dimethylaminopropyl-1-amine to obtain 0.11 g (23%) of white powder; Mp = 170–172 °C.

^{1}H NMR (600 MHz, DMSO) (δ/ppm): 9.03 (s, 1H, H_{arom}), 9.00 (t, *J* = 5.4 Hz, 1H, NH), 8.82 (t, *J* = 5.4 Hz, 1H, NH), 8.45 (s, 1H, H_{arom}), 8.28 (d, *J* = 5.5 Hz, 1H, H_{arom}), 8.16 (d, *J* = 8.5 Hz, 1H, H_{arom}), 8.7–8.05 (m, 2H, H_{arom}), 3.42–3.34 (m, 4H, CH_2), 2.32 (q, *J* = 7.1 Hz, 4H, CH_2), 2.17 (d, *J* = 3.3 Hz, 12H, CH_3), 1.76–1.71 (m, 4H, CH_2); ^{13}C NMR (75 MHz, DMSO) (δ/ppm): 166.4, 166.3, 137.9, 135.4, 134.1, 131.8, 131.6, 129.9, 129.5, 128.3, 124.9, 123.7, 123.2, 121.8, 57.4, 45.6 (2C), 38.4, 27.6, 27.5; Found: C, 65.21; H, 7.50; N, 12.51. Calc. for $C_{24}H_{32}N_4O_2S$: C, 65.42; H, 7.32; N, 12.72%.

4.2. Antiproliferative activity *in vitro*

4.2.1. Cell culturing

The newly prepared compounds were tested on five human cell lines HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), HepG2 (hepatocellular carcinoma), CFPAC-1 (ductal pancreatic adenocarcinoma) and A549 (lung adenocarcinoma) cells cultured as monolayers. The cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

4.2.2. Proliferation assays

The experiments were implemented according to the standard previously published experimental procedure. Commercial drug 5-fluorouracile was chosen as a control substance for comparison of tested effects. The calculation of IC_{50} and LC_{50} values for each compound was performed using mathematical analysis described earlier.

4.2.3. Western blot analysis

The HeLa and HepG2 cells were seeded in six well plate, $3x10^5$ cells/well, and treated with compounds **8** and **11** at concentration of $2xIC_{50}$ value for 48h and 72h respectively. Protein lysates were prepared using a buffer containing 50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease and phosphatase inhibitor cocktail (Roche, Switzerland). A total of 50 μg of proteins were resolved on 12% SDS polyacrylamide gels using the Mini-protean cell (Bio-Rad, USA).

The membranes were incubated with primary antibodies raised against hydroxy-HIF-1α (Pro564) (HIF-1α ,1:1000, rabbit mAb, Cell Signaling Technology, NL) and phospho-MEK1/2 (Ser271/221) (MEK1/2, 1:1000, rabbit mAb, Cell Signaling Technology, NL) at 4°C overnight. Secondary antibody linked to anti-mouse (1:1000, Dako, USA) was used.

The signal was visualized by Western Lightening Chemiluminescence Reagent Plus Kit (Perkin Elmer, USA) on the ImageQuant LAS500 (GE Healthcare, USA). The signal was visualized by Western Lightening Chemiluminescence Reagent Plus Kit (Perkin Elmer, USA) on the ImageQuant LAS500 (GE Healthcare, USA) and α- tubulin (1:1000, mouse mAb, Sigma, USA) was used as a loading control. The signal intensities of particular bands were normalized with the intensity of the loading control and compared in Quantity One software (Bio-Rad, USA). The values are expressed as the average ± SEM. Differences in protein relative expression status obtained by Western blot analysis were analyzed by two-tailed paired t-test ($p<0.05$) in Statistica software package (v.12.0).

4.3. Mode of action analysis

4.3.1. Dataset and pre-processing

A target/functional analysis was performed on the phenanthrene and naphtho[2,1-*b*]thiophene derivatives. Before performing our target prediction analysis, the SMILES of these derivatives were standardized using ChemAxon (v.16.5.2.0, 2016, http://www.chemaxon.com) with options “Remove Fragment” (keep largest), “Neutralize”, “RemoveExplicitH”, “Clean2D”, “Mesomerize”, and “tautomerize” for structure normalization.

4.3.2. Target prediction analysis

To predict the potential biological targets of our derivatives, PIDGIN target prediction algorithm was used. It is a Bernoulli Naïve Bayes (BNB) based method that returns, for each

input compound and for each of 1,651 potential human protein targets, the probability that the compound will have an activity on the given target (probability of activity, *Pa*). These potential human protein targets used for PIDGIN model training were extracted from ChEMBL database and because of its BNB training process PIDGIN only considered the 1651 proteins having 10 or more compounds that target them. PIDGIN was applied on of all 23 derivatives against all 1,651 potential targets, resulting in 23x1,651 = 37,973 compound-target interactions. To only consider the most probable compound-target interactions, a probability of activity (*Pa*) threshold of 0.9 (90% confidence) was chosen for the study. By using this threshold, 277 compound-target interactions between the 23 derivatives and 112 unique targets were predicted.

4.3.3. Functional analysis of the predicted targets

Further, to understand the biological role of predicted targets of our phenanthrene and naphtho[2,1-*b*]thiophene derivatives, *ClueGO* tool was used (to perform functional enrichment analysis against the background set of 1,651 human targets from PIDGIN model).[37] *ClueGO* is a plug-in of Cytoscape, which extracts representative functional information for a list of genes/proteins based on the latest publically available data from multiple annotation and ontology resources.[38] Here, *ClueGO* was used for Gene ontology (GO) Biological Process (BP), Molecular Function (MF), Cellular Component (CC) and pathway enrichment analysis. It was considered that the predicted targets are enriched in a given annotation term if the corresponding enrichment *p-value* (after Bonferroni correction for multiple hypothesis testing) is ≤ 0.05.

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Research Highlights

- substituted phenantrene and naphtho[2,1-*b*]thiophene derivatives
- antiproliferative activity on a panel of human cancer cell lines
- cyano derivatives with a pronounced and selective activity against HeLa and HepG2 cells
- mode of biological action analysis for the most active compounds performed *in silico* and *in vitro*
- Western blot analysis of HIF-1-α relative expression for most active compounds

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: