Full Paper

Synthesis, Pharmacophores, and Mechanism Study of Pyridin-2(1*H*)-one Derivatives as Regulators of Translation Initiation Factor 3A

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Twenty-seven 1,5-disubstituted-pyridin-2(1*H*)-one derivatives were synthesized and evaluated for their anti-cancer and anti-fibrosis activity by A549 and NIH3T3 cell viability assays, respectively. To study the selectivity between the cancer and fibrosis cell lines, pharmacophore models (F_1–F_4) were built in advance for compounds with pyridin-2(1*H*)-one scaffold, which revealed the relationship between the occupation of the aromatic sub-site F_4 and potent anti-cancer activity. The relationship between structure and anti-cancer activity for all target compounds is also reported herein: 1-Phenyl-5-((*m*-tolylamino)methyl)pyridine-2(1*H*)-one (**22**) displayed both potency and selectivity ($IC_{50} = 0.13$ mM) toward the A549 cell line through the inhibition of translation initiation, especially by eIF3a suppression, and can be treated as a lead for the design of novel eIF3a regulators and anti-lung cancer agents.

Keywords: Eukaryotic translation initiation factor 3, subunit A (eIF3a) / Fibrosis / Lung cancer / Pyridin-2(1*H*)-one / Regulators

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Introduction

Lung cancer is the leading cause of cancer-related mortality with an estimated yearly incidence of 1.2 million new cases worldwide and its 5-year overall survival rate is approximately 16% [1]. The low efficiency of conventional therapies in achieving long-term survival of patients with lung cancer calls for the development of novel treatment/prevention options. In this regard, chemoprevention using naturally occurring or synthetic compounds to arrest or reverse the carcinogenic process maybe extremely important as a lung cancer prevention strategy [2].

Protein synthesis in eukaryotes is a complicated process that involves many eukaryotic translation initiation factors (eIFs). In eukaryotes, at least 13 factors have been identified to participate in the translation initiation step [3, 4]. Among these factors, eIF3 is the largest and the most complex one with molecular weight of about 550–700 kDa [3, 4]. eIF3a is the largest subunit of eIF3 and has been suggested to play critical roles in regulating synthesis of proteins including α-tubulin, ribonucleotide reductase M2, and p27 as well as in regulating cell cycle progression and cell proliferation and differentiation [5–8]. Nevertheless, eIF3a is essential for cancer cells to maintain malignant phenotype [6] and ectopic over-expression of eIF3a transformed NIH3T3 cells *in vitro* [9]. Although over-expression of eIF3a has been found in many cancers such as cancers of breast [10], cervix [11], stomach [12], and esophagus [13], eIF3a appeared to be specifically over-expressed in human lung cancer and played vital roles in pathogenesis and prognosis of this disease [14, 15]. Furthermore, it is discovered that lung cancer cell is more sensitive to cisplatin treatment due to higher levels of eIF3a, which further reveal the close relationship between eIF3a and lung cancer. eIF3a also regulates the expression and activity of

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nucleotide excision repair (NER) proteins, which mediate the effect of eIF3a on cellular response to DNA-damaging anti-cancer drugs [15]. Therefore, eIF3a is recognized as an attractive bio-marker in the treatment of proliferative diseases, especially lung cancer.

However, little is known about small molecule eIF3a regulators up to date. The only compound reported is L-mimosine, a plant amino acid, which was developed as cell cycle blocker (Fig. 1). L-Mimosine can specifically and reversibly block mammalian cells at late G1 phase [16]. Recently, it has been suggested that eIF3a acts as a mediator of L-mimosine effect on the protein synthesis [5]. The decreased expression of eIF3a by L-mimosine is due to its iron-chelating function of 3-hydroxy-4-carbonyl group, which results in up-regulated expression of CDK inhibitor (p27), down-regulated expression of ribonucleotide reductase M2 [6] and *de novo* synthesis of tyrosinated α-tubulin [5].

Additionally, as an analog, pyridin-2(1*H*)-one scaffold is also widely applied in the design of novel drugs because of its potency of anti-inflammatory [17] and anti-fibrosis activity [18, 19]. In recent years, it is suggested that pulmonary fibrosis share similar pathological ground to lung cancer, which is mediated by growth factors [20] and is associated with an increased risk of lung cancer [21, 22]. Another pyridin-2(1*H*)-one derivative, 5-methyl-1-phenylpyridin-2(1*H*)-one (pirfenidone, PFD) is the only small molecular drug coming into market for anti-fibrosis therapy (Fig. 1). In our previous efforts to study the mechanism and modification of PFD, we discovered 1-(3-fluorophenyl)-5-methylpyridin-2(1*H*)-one (fluorofenidone, AKF-PD), which shows better anti-fibrosis activity than that of PFD (Fig. 1) [23, 24]. In recent studies, PFD was reported to display antitumor effects on malignant gliomas by inhibition of the expression of TGF-β [25] and EGFR phosphorylation [26].

To further conduct structure modification and anti-proliferation activity study based on the pyridin-2(1*H*)-one scaffold, we designed and synthesized a novel series of 1,5-disubstituted-pyridone derivatives using PFD as a lead (Fig. 2). *In vitro* cell viability assays were conducted on human A549 and mice NIH3T3 cell lines to evaluate their anti-cancer and anti-fibrosis activity and selectivity using MTT methods. Pharmacophore models were also built using Molecular Operating Environment (MOE, Chemical Computing Group, Inc., Canada) to elucidate differences on selectivity. Furthermore, to further evaluate whether eIF3a protein was involved in the effect of potent compounds against cell proliferation, the levels of eIF3a mRNA and protein upon drug treatment were determined by real-time PCR and immunoblot analysis assay in cancer cell line.

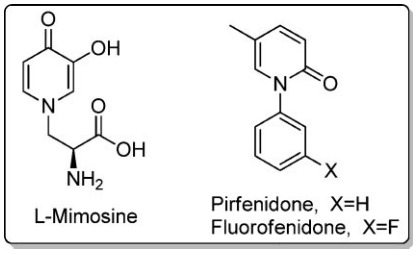


Figure 1. The chemical structures of L-mimosine, pifenidone (PFD), and fluorofenidone (AKF-PD).

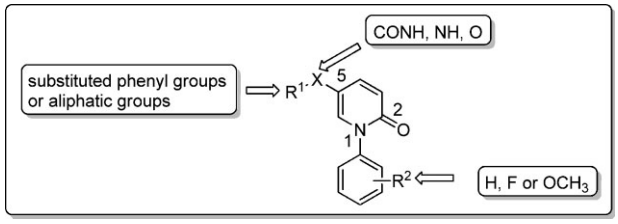


Figure 2. Designing strategy for pyridin-2(*1H*)-one derivative.

Chemistry

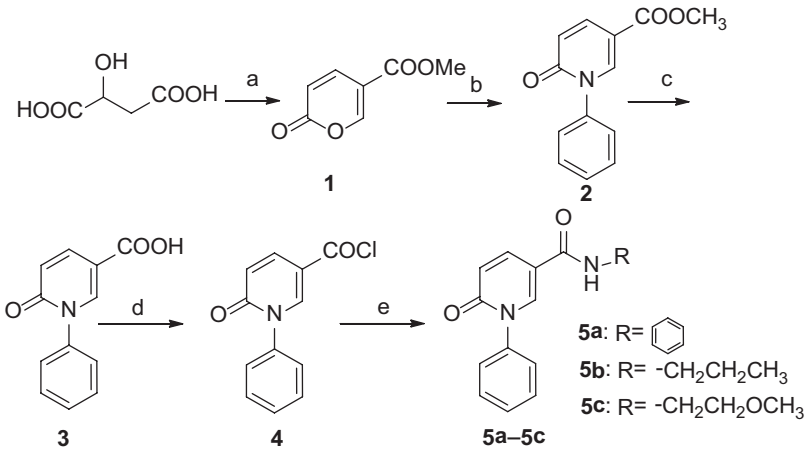
Amides **5a**–**5c** were synthesized from malic acid following the methods reported previously as shown in Scheme 1 [27]. In brief, compound **1** was prepared in high yield and treated with aniline in ethanol to obtain 1-substituded pyridin-2(1*H*)-one **2** in the form of ester [28], which was further hydrolyzed and acidified to obtain acid **3**. The final compound **5** was obtained following classical methods for the synthesis of amides under basic condition from acyl chloride **4**.

The synthetic procedures for amines (compounds **9**–**29**) and ethers (compounds **30**–**32**) consisted of two main reactions: Ullmann reaction [29, 30] and bromination reaction [31, 32] as illustrated in Scheme 2 to yield intermediates **7a**–**7c**, which were finally SN_2 substituted with different aniline derivatives or sodium salt of substituted phenol in acetonitrile.

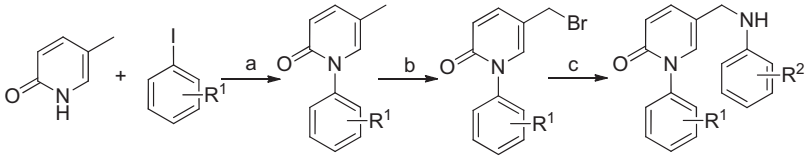
Additionally, other than to obtain **7a**, the bromination reaction for **6a** will also yield products **7e** and **7f** because C-3 is also active under the same radical conditions, and **7d** as well, which is consistent with [31]. This can be further confirmed by the purification of **8a** from the mixture of bromination reaction for **6a** with the treatment of CH_3ONa. Furthermore, during the synthesis of compound **9**, C-5 aldehyde derivative **8b** was also obtained from the hydrolysis of imine formed between C-5 and benzyl group because of the di-brominated product **7d** (Scheme 3) [33].

Results and discussion

To investigate the anti-proliferation activities of pyridin-2(1*H*)-one derivatives, the *in vitro* cell viability assay was conducted



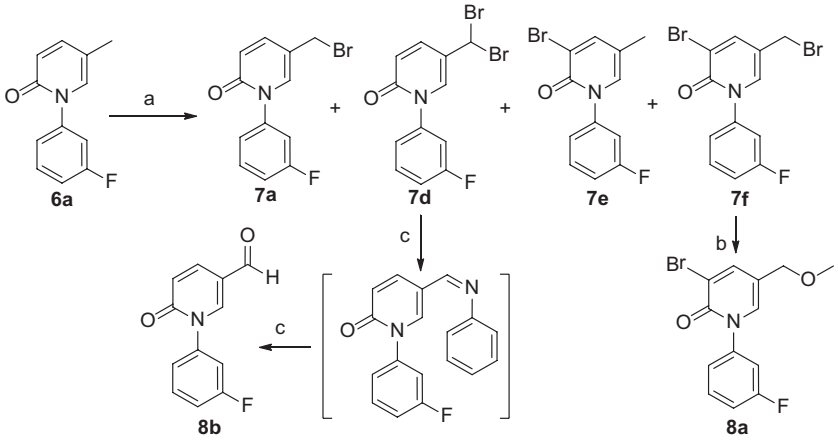
Scheme 1. Reagents and conditions: (a) (i) H_2SO_4, SO_3, 98–104°C, 1.5 h; (ii) CH_3OH, 95–100°C, 2 h, 65%; (b) (i) aniline, C_2H_5OH; (ii) 15% Na_2CO_3, 73%; (c) (i) 10% NaOH, 3 h; (ii) 5% HCl, 87%; (d) $SOCl_2$, reflux, 4 h; (e) RNH_2, CH_2Cl_2, reflux, 4 h.



6a,7a: R^1= *m*-F
6b,7b: R^1=H
6c,7c: R^1=*p*-OMe

9: R^1=*m*-F,R^2=H
10: R^1=*m*-F,R^2=*p*-COMe
11: R^1=*m*-F,R^2=*p*-Me
12: R^1=*m*-F,R^2=*p*-OMe
13: R^1=*m*-F,R^2=*m*-Me
14: R^1=*m*-F,R^2=*o*-Me
15: R^1=m-F,R^2=*p*-Cl
16: R^1=H,R^2=*o*-Me
17: R^1=H,R^2=*p*-Me
18: R^1=H,R^2=*p*-OMe
19: R^1=H,R^2=*p*-COMe
20: R^1=H,R^2=H
21: R^1=H,R^2=*p*-Cl
22: R^1=H,R^2=*m*-Me
23: R^1=*p*-OMe,R^2=*p*-Cl
24: R^1=*p*-OMe,R^2=*p*-COMe
25: R^1=*p*-OMe,R^2=*p*-OMe
26: R^1=*p*-OMe,R^2=H
27: R^1=*p*-OMe,R^2=*p*-Me
28: R^1=*p*-OMe,R^2=*o*-Me
29: R^1=*p*-OMe,R^2=*m*-Me

Scheme 2. Reagents and conditions: (a) Cu, K_2CO_3, DMF, 160–170°C, reflux, 20 h, 61–75%; (b) *N*-bromosuccinimide (NBS), azobisisobutyronitrile (AIBN), CCl_4, medium-pressure mercury (450-W), reflux, 4 h; (c) substituted aniline, CH_3CN, r.t., 2.5 h.



Scheme 3. Reagents and conditions: (a) *N*-bromosuccinimide (NBS), azobisisobutyronitrile (AIBN), CCl_4, medium-pressure mercury (450-W), reflux, 4 h; (b) *m*-fluoroaniline, CH_3CN, r.t., 2.5 h; c. CH_3OH, NaOH.

on two cell lines with different states of eIF3a expression: A549, a human lung adenocarcinoma cell with high expression of eIF3a, and NIH3T3, a mouse embryonic fibroblast cell line with low expression of eIF3a. Cisplatin and PFD were used as control. The results for compounds **5** and **9** are shown in Table 1.

Structure–selectivity relationship (SSR) for pyridin-2(1*H*)-one derivatives with different substituents at R_2

*Cell viability assays for compound **5** and **9***

PFD was developed as an anti-fibrosis agent and, in recent years, it was further reported to display antitumor effects on malignant glioma cells. In order to get insight into the influencing factors on the selectivity of pyridin-2(1*H*)-one derivatives toward different cells, aromatic ring or aliphatic chain were introduced into substituents at R_2 (Scheme 1). As shown in Table 1, PFD shows good selectivity against NIH3T3 cell line which is consistent with its anti-fibrosis application, whereas compound **5a** reversed the selectivity from fibroblast cell to cancer cell, which show 63 times selectivity value toward A549 cell line compared to that of PFD. When compared with compounds with aliphatic substituents at R_2 (**5b**, **5c**), the experimental data for those with aromatic substituents at R_2 (**5a**, **9**) demonstrated that the existence of aromatic ring at R_2 is more favorable and selective for the inhibition of cancer cell proliferation.

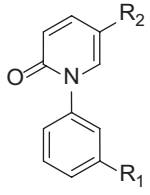
*Selectivity study based on pharmacophore building for PFD, **5a** and **9***

Herein, to get further insight into the changes in selectivity based on structure modification, **5a** and **9** were aligned and the 4-pharmacophore query (F_1–F_4) was built using flexible alignment and pharmacophore query within MOE as shown in Fig. 3a. Compound **5a** and **9** shared similar F_4 occupation (Fig. 3b). However, F_4 aromatic/hydrophobic feature cannot be occupied by PFD due to its vacancy on the C-5 position of pyridin-2(1*H*)-one, which suggest the critical role in the selectivity profiles for F_4 occupation (Fig. 3c).

*Western blot assay for compounds **5**, **9**, and AKF-PD*

As mentioned before, eIF3a is a specific bio-marker of lung cancer. The possible reason for the selectivity change for pyridin-2(1*H*)-one derivatives may partially be due to the over-expression of eIF3a in lung cancer, which can extremely increase the sensitivity of anti-cancer agents toward A549 cell line as described previously [24]. To further evaluate whether eIF3a is involved in the effect of novel pyridin-2(1*H*)-one derivatives, we next examined the expression levels of eIF3a protein upon treatment with **5a** (C-5 amide derivatives), **9** (C-5 amine derivatives), and AKF-PD in A549 cells by Western blot methods as described previously [15]. As shown in Fig. 4, AKF-PD (with no substituent at R_1 on C-5 position) exhibited no inhibitory effects on eIF3a expression, whereas **5a** and **9** significantly and consistently suppressed the eIF3a level at 600 μM, which means that F_4 occupation is a necessary requirement for the inhibition of eIF3a expression. Secondly, the existence of C-5 aromatic substituent occupying F_4 sub-site is more favorable for eIF3a inhibition for amine analogs (**9**, with inhibitory rate of 80%) than for amide group (**5a**, with inhibitory rate of 30%), which make amine derivatives with aromatic ring attractive to develop more potent and selective anti-cancer agents as eIF3a regulators.

Table 1. *In vitro* cell viability assay results for compound **5** and **9**



	Substituents		IC_{50} (mM)[a]		Ratio of values	
NCE	R_1	R_2	NIH3T3	A549	NIA	NCP
5a	H	−CONHPh	564.32 ± 25.32	31.5 ± 12.34	17.33	64.15
5b	H	$-CONHC_3H_7$	3.47 ± 1.25	8.08 ± 1.97	0.43	1.59
5c	H	$-CONH(CH_2)_2OCH_3$	6.47 ± 1.46	14.08 ± 2.79	0.46	1.70
9	F	$-CH_2NHPh$	0.39 ± 0.12	0.46 ± 0.19	0.85	3.14
PFD	H	$-CH_3$	2.27 ± 0.58	8.32 ± 2.53	0.27	1.00
Cisplatin			0.015 ± 0.007	0.06 ± 0.01	0.25	0.93

[a] The data shown are representative of three individual experiments. NIA = $IC_{50(NIH3T3)}/IC_{50(A549)}$; NCP = ratio $IC_{50(NCE)}$/ratio $IC_{50(PFD)}$.

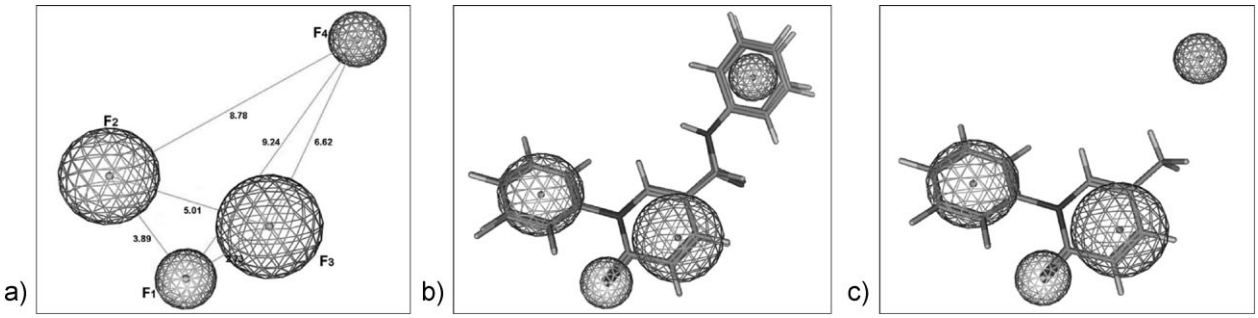


Figure 3. Selectivity study based on pharmacophore modeling: (a) 4-point pharmacophore features built from **5a** and **9** (F_1: hydrogen bond acceptor; F_2–F_4: hydrophobic or aromatic sub-site); (b) alignment for compounds **5a** and **9** to pharmacophore features; (c) alignment for PFD with pharmacophore features. All the models were built using MOE software.

Structure–activity relationship (SAR) for pyridin-2(1*H*)-one derivatives 10–32

To further obtain novel chemical entries with more structure varieties occupying F_4 sub-site and with higher selectivity and potency, the R_2 substituent on C-5 position of pyridin-2(1*H*)-one was modified to substituted aniline (**10**–**29**, Scheme 2). As shown in Table 2, most compounds showed strong inhibitory activity against both cell lines with IC_{50} values of 0.13–14.50 mM. However, the selectivity toward A549 was retained, which confirmed that the higher level expression of eIF3a in lung cancer results in more sensitive effect for pyridin-2(1*H*)-one derivatives with F_4 occupation.

Structure–activity relationship (SAR) study indicated that both anti-fibrosis and anti-cancer activity for pyridin-2(1*H*)-one derivatives are not related with types of substituents at R_1 or R_2. Both **21** and **22** displayed better selectivity and potency toward A549 cell line. The most potent compound **22** displayed similar activity (IC_{50} = 0.13 mM) to that of cisplatin (IC_{50} = 0.06 mM). Additionally, what is most notable for compound **22** is that this compound possesses highest selectivity toward A549 cell line compared with PFD, which shows 79.3 times for IC_{50} ratio value and makes it the most selective and potent inhibitor with pyridin-2(1*H*)-one scaffold for lung cancer cell line up to now.

To further study whether the hydrogen bond donor (NH) on C-5 position is important for the activity profile, the NH group was isosterically replaced by oxygen to obtain a series of 1-substituted phenyl-5-substituted phenyloxymethyl-2(1*H*)-pyridinone. What was interesting is that compound **30** and **31** displayed most potency against NIH3T3 cell proliferation with IC_{50} value of 0.016 and 0.014 mM, respectively (Table 3). This suggested that the hydrogen bond acceptor (O) on C-5 position may be more favorable for the anti-fibrosis activity of pyridin-2(1*H*)-one. Regardless of the loss of selectivity for compound **30**, however, it still arouse our interests in its anti-fibrosis activity and further structure modification work about compound **30** in our group is under the way.

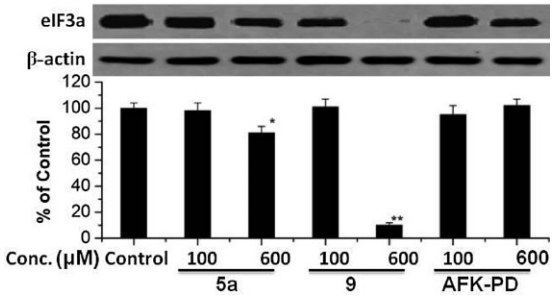


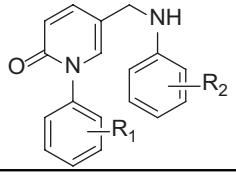
Figure 4. Western blot analysis results for pyridin-2(*1H*)-one derivatives (**5a**, **9**, and AKF-PD) in A549 cells. Each bar represents the mean ± SD of at least three different experiments. The bars for eIF3a protein are plotted with the percentage about ratios value of eIF3a/β-actin of DMSO control.

Suppression of eIF3a expression for L-mimosine and pyridin-2(1*H*)-one derivatives

Dysregulation of protein synthesis, especially hyperactivity in translation or post-translational modification procession, is deeply involved in tumorigenesis and therapeutic effects of cancer and restoration of translation regulation provide a promising pattern for cancer therapy [34]. Up to date, there are quite a few of antitumor agents suppressing translation including retinoic acid, rapamycin, wortmannin, homoharringtonine, bruceantin, and didemnin B, which down-regulate translation through different mechanisms [35–37]. Additionally, Dong and co-workers reported L-mimosine (Fig. 1) inhibited protein synthesis during initiation of translation, which is associated with the inhibition of protein synthesis of eIF3a [5].

To further evaluate the mechanism of action on the inhibition of eIF3a expression (whether transcription or post-translational level) for pyridin-2(1*H*)-one derivatives, Western blot analysis and real-time PCR analysis in A549 cells were accessed for novel active compounds in different

Table 2. *In vitro* cell viability assay results for compound **10**–**29**.



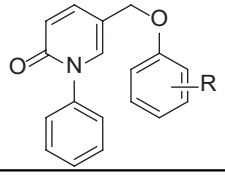
	Substituents		IC_{50} (mM)[a]		Ratio of values	
NCE	R_1	R_2	NIH3T3	A549	NIA	NCP
10	*m*-F	*p*-COMe	7.20 ± 1.89	7.06 ± 2.34	1.02	3.78
11	*m*-F	*p*-Me	28.40 ± 3.49	–	–	–
12	*m*-F	*p*-OMe	147.10 ± 5.74	5.39 ± 0.97	27.29	101.07
13	*m*-F	*m*-Me	0.36 ± 0.15	0.38 ± 0.24	0.95	3.52
14	*m*-F	*o*-Me	0.34 ± 0.08	0.29 ± 0.19	1.17	4.33
15	*m*-F	*p*-Cl	5.10 ± 1.09	14.50 ± 2.56	0.35	1.29
16	H	*o*-Me	0.41 ± 0.21	0.32 ± 0.07	1.28	4.74
17	H	*p*-Me	0.23 ± 0.04	0.28 ± 0.03	0.82	3.04
18	H	*p*-OMe	1.57 ± 0.59	1.01 ± 0.21	1.55	5.74
19	H	*p*-COMe	1.16 ± 0.97	5.92 ± 1.11	0.20	0.74
20	H	H	3.50 ± 1.59	0.80 ± 0.32	4.38	16.22
21	H	*p*-Cl	4.09 ± 1.67	0.32 ± 0.13	12.78	47.33
22	H	*m*-Me	1.52 ± 0.37	0.13 ± 0.04	11.69	43.29
23	*p*-OMe	*p*-Cl	3.75 ± 0.88	2.17 ± 0.34	1.72	6.37
24	*p*-OMe	*p*-COMe	0.57 ± 0.27	0.95 ± 0.06	0.6	2.22
25	*p*-OMe	*p*-OMe	3.18 ± 0.89	1.51 ± 0.12	2.11	7.81
26	*p*-OMe	H	2.54 ± 1.23	1.70 ± 0.38	1.49	5.52
27	*p*-OMe	*p*-Me	3.52 ± 2.64	1.53 ± 0.64	2.30	8.52
28	*p*-OMe	*o*-Me	1.63 ± 0.59	1.91 ± 0.31	0.85	3.15
29	*p*-OMe	*m*-Me	21.34 ± 3.49	1.92 ± 0.22	11.11	41.14

[a] The data shown are representative of three individual experiments. NIA = $IC_{50(NIH3T3)}/IC_{50(A549)}$; NCP = ratio $IC_{50(NCE)}$/ratio $IC_{50(PFD)}$.

concentrations: PFD in high concentration (300 μM), **22** and L-mimosine (200 μM), and **30** in low concentration (100 μM) using conventional methods as described previously [15].

As shown in Fig. 5, after incubation of A549 cell with different compounds for 8 h, L-mimosine and PFD in high concentration caused a decrease in mRNA levels of eIF3a to about a half of control whereas compound **22** and **30** in low concentration displayed less influence to the levels of eIF3a mRNA. However, what interested us was that the suppression of protein did not proportionate with the level of mRNA, which showed opposite results: the eIF3a protein level of A549 cells were down-regulated by **22** and **30** substantively in low

Table 3. *In vitro* cell viability assay results for compound **30**–**32**.



	Substituents	IC_{50} (mM)[a]		Ratio of values	
NCE	R	NIH3T3	A549	NIA	NCP
30	*m*-Me	0.016 ± 0.002	0.28 ± 0.13	0.06	0.21
31	*p*-Cl	0.014 ± 0.003	0.22 ± 0.10	0.06	0.24
32	H	0.043 ± 0.005	0.38 ± 0.12	0.11	0.41

[a] The data shown are representative of three individual experiments. NIA = $IC_{50(NIH3T3)}/IC_{50(A549)}$; NCP = ratio $IC_{50(NCE)}$/ratio $IC_{50(PFD)}$.

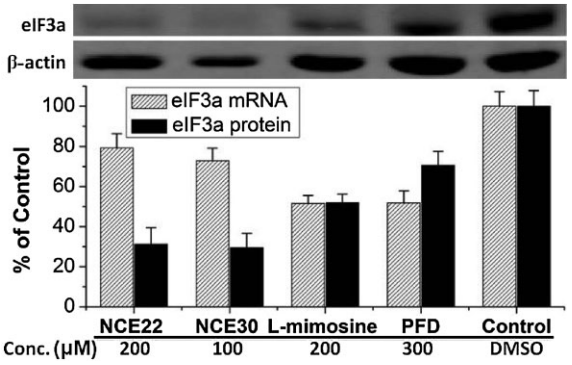


Figure 5. Western blot analysis and mRNA RT-PCR results for pyridin-2(*1H*)-one derivatives (PFD, **22**, **30**) and L-mimosine in A549 cells. Each bar represents the mean ± SD of at least three different experiments. The bars for eIF3a protein are plotted with the percentage about ratios value of eIF3a/β-actin of DMSO control. The bars for mRNA levels are plotted with the percentage of DMSO control.

concentration, whereas L-mimosine and PFD in high concentration displayed less effect on the protein level. The interesting results indicated a post-translational level about the suppression of eIF3a expression for pyridin-2(1*H*)-one derivative (**22** and **30**), which is a quite different manner from that of L-mimosine demonstrated previously to be a transcription inhibitor of eIF3a expression.

These results indicated that the mechanism of inhibition on protein synthesis by pyridin-2(1*H*)-one derivative is also much distinct from the known inhibitors of protein synthesis, which is likely to be operating at the initiation phase of translation through eIF3a. Additionally, pyridin-2(1*H*)-one may not be fully functional of pyridin-4(1*H*)-one as exemplified by L-mimosine. Unraveling this mechanism may open a window for new therapeutic opportunities for the treatment of tumors, especially lung cancer. Pyridin-2(1*H*)-one derivatives may also be used in combination with therapeutic agents that show different mechanisms of action and multi-targeted therapeutic strategy, as well as overcoming the drug resistance characteristics.

Conclusion

A novel series of PFD analogs with different substitutions on the N-1 position and C-5 position of pyridin-2(1*H*)-one scaffold were designed and synthesized to conduct structure–selectivity relationship (SSR) and SAR as well as mechanism studies.

Cell-based *in vitro* viability assays demonstrated that F_4 occupation can improve the sensitivity of most compounds to lung cancer cell line and change the selectivity of PFD analogs. The reasons lie in: (i) eIF3a is a specific bio-marker for the sensitivity of lung cancer cell line, which is superior to fibroblast cell line; (ii) pyridin-2(1*H*)-one derivative, especially compounds with F_4 occupation, demonstrates better anti-proliferation activity. Of all the three series derivatives, compound **22** most selectively and effectually inhibits the cancer cell proliferation; (iii) the functional activities of active compounds with F_4 occupation are associated, at least partially, with their inhibition of translation initiation by suppressing eIF3a as demonstrated by RT-PCR and Western blot analysis.

In summary, given the important roles of eIF3a protein in cell growth, development and tumorigenesis, for the first time our studies suggest that eIF3a is an attractive therapeutic target in the treatment of lung cancer and also demonstrate the relationship between the F_4 occupation and eIF3a protein level, which are important for selectivity toward cancer cells. The structure with F_4 occupation especially 1-substitued phenyl-5-((substituted phenylamino)methyl)pyridine-2(1*H*)-one derivatives can be considered as a promising scaffold to develop small molecule eIF3a regulators as potential anti-lung cancer agents.

Experimental

Chemistry

All chemicals and solvents were used as received from commercial sources without further treatment unless otherwise stated. All reactions were monitored by TLC with 0.25 mm silica gel plates (60GF-254). UV light was used to visualize the spots. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DRX 400 NMR instruments with TMS as an internal standard, δ in parts per million and J in Hertz. Mass spectra were recorded on Qstar LC/MS instrument. Melting points were determined uncorrected on a BUCHI electrothermal melting point apparatus. Column chromatography was performed with commercial silica gel (300–400 mesh) for purification. All tested compounds are >95% pure by HPLC analysis performed on an Agilent 1100 HPLC instrument using an Agela Promosil C18 column (250 mm × 4.6 mm, 5 μm) according to one of the following methods over 20 min, with detection at 254 nm and a flow rate of 1.0 mL/min. *Method A*: Compounds **5a–5c**, **9–12**, **18–20**, and **23–25** were eluted with 60% methanol/40% water. *Method B*: Compounds **13–17**, **21–22**, and **27** were eluted with 65% methanol/35% water. *Method C*: Compounds **26** and **28–32** were eluted with 70% methanol/30% water.

General procedure for the preparation of C-5 amides derivatives (5a–5c)

*Synthesis of methylcoumalate (**1**)*

To 134 g of malic acid was added 155 mL of concentrated sulfuric acid followed by 70 mL of 50% oleum. The resulting slurry was heated at 98–104°C for 1.5 h. The solution was cooled to room temperature and 170 mL of dry methanol was added slowly. The resulting solution was heated at 95–100°C for 2 h, allowed to cool to room temperature and then poured into 120 mL of cold water. After standing for 0.5 h, the solution was extracted with chloroform and upon evaporation of the solvent, recrystallization

from EtOAc to give compound **1**. Slight yellow solid, 65% yield. mp: 68.9–70.7°C ([38]; 65–66°C).

*Synthesis of methyl 6-oxo-1-phenyl-1,6-dihydropyridine-3-carboxylate (**2**)*

11.2 g of aniline was added slowly to a solution of 15.4 g of methylcoumalate in 150 mL of alcohol, after stirring for a few minutes, crystallization of a yellow solid occurred, which was filtered-off and dried for 4 h under vacuum at 45°C. The crude solid was dissolved in 140 mL of 15% Na_2CO_3, heated to 40–45°C, cooled, and the solid formed was filtered-off and recrystallized from isopropyl alcohol to obtain **2**. White solid, 73% yield. mp: 198.7–200.4°C ([28]; 100–105°C).

*Synthesis of 6-oxo-1-phenyl-1,6-dihydropyridine-3-carboxylic acid (**3**)*

One gram of methyl 6-oxo-1-phenyl-1,6-dihydropyridine-3-carboxylate (**2**) was heated at 50°C and dissolved in 32 mL of 10% NaOH, cooled, and stirred for 3 h. The solution's pH was modulated by diluted HCl to 2–3 to form white solid, which was filtered-off and recrystallized from methanol. White solid, 87% yield. mp: 218.5–220.0°C.

*Synthesis of 6-oxo-N,1-diphenyl-1,6-dihydropyridine-3-carboxamide (**5a**)*

4.3 g (0.02 mol) of **3** was added and dissolved to 50 mL $SOCl_2$, refluxed for 4 h. To the slight yellow oil obtained after complete removal of $SOCl_2$ was added 30 mL of CH_2Cl_2, 3.7 g (0.04 mol) of aniline. After heating at reflux for 4 h, the solvent was removed and the crude product of **5a** was purified upon column chromatography (EtOAc/petroleum ether). White solid, 69% yield. HPLC: Rt = 7.48 min, 99.4%. mp: 196.1–197.8°C. IR (KBr, cm^{-1}): 3340, 3051, 1663, 1608, 1597, 1523, 1493, 1483, 1327, 1276, 1128. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.64 (d, 1H, J = 9.6 Hz), 7.14 (s, 1H), 7.30 (m, 3H), 7.48 (m, 4H), 7.55 (d, 2H, J = 8.0 Hz), 7.85 (d, 1H, J = 9.6 Hz), 8.02 (d, 1H, J = 9.6 Hz), 8.17 (d, 1H, J = 2.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 114.2, 120.4, 120.5 (2C), 124.7, 126.4, 129.0 (2C), 129.2, 129.5, 137.7, 140.1, 141.3, 162.1, 162.2. MS m/z: 290 $[M]^+$.

*Synthesis of 6-oxo-1-phenyl-N-propyl-1,6-dihydropyridine-3-carboxamide (**5b**)*

Following the same procedure as reported for the synthesis of **5a**. White solid, 65% yield. HPLC: Rt = 4.44 min, 98.9%. mp: 121.7–123.5°C. IR (KBr, cm^{-1}): 3348, 3073, 2970, 2926, 2874, 1682, 1652, 1608, 1589, 1519, 1486, 1360, 1316, 1294, 1153, 1116. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.92 (m, 3H), 1.52 (m, 2H), 3.25 (m, 2H), 6.51 (m, 2H), 7.33 (m, 2H), 7.47 (m, 3H), 7.72 (m, 1H), 8.09 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.4, 22.8, 41.7, 113.8, 120.3, 126.4 (2C), 129.0, 129.4 (2C), 137.3, 140.2, 140.7, 162.0, 163.8. MS m/z: 256 $[M]^+$.

*Synthesis of N-(2-methoxyethyl)-6-oxo-1-phenyl-1,6-dihydropyridine-3-carboxamide (**5c**)*

White solid, 60% yield. HPLC: Rt = 6.68 min, 99.6%. mp: 116.8–118.4°C. IR (KBr, cm^{-1}): 3325, 3066, 2948, 2836, 1671, 1637, 1527, 1316, 1260, 1194, 1153, 1120. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.35 (s, 3H), 3.53 (t, 2H), 3.56 (m, 2H), 6.60 (d, 2H, J = 7.2 Hz), 7.35 (d, 2H, J = 8.8 Hz), 7.47 (m, 3H), 7.72 (dd, 1H, J = 9.6 Hz, 2.4 Hz), 8.10 (d, 1H, J = 2.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.6, 58.7, 70.9, 113.4, 120.5, 126.4, 129.0, 129.4 (2C), 137.1, 140.2, 140.8, 162.0, 163.9. MS m/z: 272 $[M]^+$.

General procedure for the synthesis of C-5 amines derivatives (9–29)

*Synthesis of 1-(3-fluorophenyl)-5-methylpyridin-2(1H)-one (**6a**)*

To 9.6 g of anhydrous DMF was added 45.0 g (0.2 mol) of 1-fluoro-3-iodophenyl, 21.8 g (0.2 mol) of 5-methyl-2-pyridone, 28 g of K_2CO_3 and 1.0 g of copper. The mixture was stirred and heated to 160–170°C and kept refluxing for 20 h. When the suspension was cooled naturally to 110–120°C, about 330 mL of hot water (80–90°C) was slowly poured in. Reaction mixture was cooled to 20°C and filtered; residue was dissolved in 300 mL ethyl acetate ultrasonically. After the filtration, the solid cake was washed with ethyl acetate (20 mL × 2) and the combined ethyl acetate phase was washed with water (50 mL × 3). The organic layer was evaporated and to the residues was added 85 mL of hot petroleum ether and then cooled to 15–20°C. The temperature was kept for 1.5 h and the mixture was filtered to obtain crude product of **6a**, which was recrystallized from 20% ethanol. White solid, 75% yield. mp: 132.1–133.7°C.

*Synthesis of 5-methyl-1-phenylpyridin-2(1H)-one (**6b**)*

Following the same procedure as reported for the synthesis of **6a** and recrystallized from water. White solid, 73% yield. mp: 108.9–110.6°C.

*Synthesis of 1-(4-methoxyphenyl)-5-methylpyridin-2(1H)-one (**6c**)*

Following the same procedure as reported for the synthesis of **6a** and purified upon column chromatography (EtOAc/petroleum ether). White solid, 61% yield. HPLC: Rt = 18.42 min, 99.3%. mp: 101.8–103.8°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.03 (s, 3H), 3.79 (s, 3H), 6.39 (d, 1H, J = 9.2 Hz), 7.02 (d, 2H, J = 8.8 Hz), 7.2 8 (d, 2H, J = 8.8 Hz), 7.35 (dd, 1H, J = 9.2 Hz, 2.4 Hz), 7.38 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.3, 54.4, 113.8, 114.1 (2C), 120.1, 127.9, 133.9 (2C), 136.5, 142.9, 158.6, 160.6. MS m/z: 216 $[M+H]^+$.

*Synthesis of 1-(3-fluorophenyl)-5-((phenylamino)methyl)-pyridin-2(1H)-one (**9**)*

In a dried flask was placed 0.01 mol of **6a** and 150 mL of anhydrous CCl_4. To the solution was added 0.01 mol *N*-bromosuccinimide, and 0.5 g of azobisisobutyronitrile. The mixture was heated at reflux under the irradiation of 450-W lamp for 4 h. When cooled to room temperature, the mixture was filtered and the filtrate was evaporated to obtain **7a** in yellow oil. To the oil of **7a** was added 60 mL CH_3CN followed by 0.02 mol of aniline. The mixture was stirred air tightly at room temperature for 2.5 h, and then filtered. The filtrate was evaporated to a colorful solid mixture and upon column chromatography (EtOAc/petroleum ether) to obtain **9**.

Slight yellow solid, 25% yield. HPLC: Rt = 9.13 min, 97.3%. mp: 124.9–126.5°C; IR (KBr, cm^{-1}): 3059, 1523, 1490, 1449, 1312, 1286, 1264, 1235, 1153, 1113. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.00 (s, 1H), 4.12 (s, 2H), 6.66 (dd, 3H, J = 9.6 Hz, 12.4 Hz), 6.77 (t, 1H, J = 7.2 Hz), 7.14 (m, 3H), 7.20 (t, 2H, J = 8.0 Hz), 7.30 (d, 1H, J = 1.6 Hz), 7.43 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 45.0, 113.1

(2C), 114.4–114.6 (F-Ar), 115.6–115.8 (F-Ar), 117.2, 118.4, 122.2, 122.3 (F-Ar), 129.4 (2C), 130.5–130.6 (F-Ar), 135.4, 140.4, 142.0–142.1 (F-Ar), 147.4, 161, 161.8–163.9 (F-Ar). MS *m/z*: 294 $[M+H]^+$.

*1-(3-Fluorophenyl)-6-oxo-1,6-dihydropyridine-3-carbaldehyde (**8a**)*

Light yellow solid, 7% yield. HPLC: Rt = 5.11 min, 98.3%. mp: 166.6–170.2°C. IR (KBr, cm^{-1}): 3311, 3118, 3022, 1667, 1604, 1586, 1523, 1497, 1482, 1449, 1320, 1286, 1253, 1224, 1150, 1127. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.70–6.73 (d, 1H, J = 9.6 Hz), 7.17–7.27 (m, 3H), 7.50–7.56 (m, 1H), 7.88–7.91 (dd, 1H, J = 9.6, 2.4 Hz), 7.96–7.97 (d, 1H, J = 2.4 Hz), 9.69 (s, 1H, CHO). ^{13}C NMR (400 MHz, $CDCl_3$): δ 114.4–114.6 (F-Ar), 116.6, 116.8 (F-Ar), 118.5, 122.0, 122.2 (F-Ar), 122.2, 131.0, 131.1 (F-Ar), 136.1, 140.8, 140.9 (F-Ar), 146.7, 161.5, 161.9, 164.0 (F-Ar), 185.9 (CHO). MS *m/z*: 217 $[M]^+$.

*3-Bromo-1-(3-fluorophenyl)-5-(methoxymethyl)pyridin-2-(1H)-one (**8b**)*

In a dried flask was placed 0.1 mol of **6b** and 1500 mL of dry CCl_4. To the solution was added 0.1 mol of NBS and 0.5 g of AIBN. The mixture was heated at reflux with a 450-W lamp for 4 h. Then 0.05 mol NBS was added again and refluxed for one more hour. When cooled to room temperature, the mixture was filtered, the filtrate was evaporated to a yellow oil, which was dissolved in 60 mL CH_3OH, refluxed for 4 h, cooled below 10°C, standing overnight. Crystal occurred, **8b** was obtained by filtering [33].

White solid, 3% yield. HPLC: Rt = 4.27 min, 98.9%. mp: 82.3–84.2°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.40 (s, 3H), 4.21 (s, 2H), 7.16 (m, 3H), 7.32 (t, 1H), 7.46 (m, 1H), 7.84 (d, 1H, J = 2.4 Hz). ^{13}C NMR (100 MHz, CDCl3): δ 58.3, 70.6, 114.3–114.5 (F-Ar), 115.9–116.1 (F-Ar), 116.5, 117.7, 122.2 (F-Ar), 130.6–130.7 (F-Ar), 135.4, 141.8–141.9 (F-Ar), 142.4, 158.1, 161.4–163.8 (F-Ar). MS *m/z*: 312 $[M]^+$.

*5-((4-Acetylphenylamino)methyl)-1-(3-fluorophenyl)-pyridin-2(1H)-one (**10**)*

Tawny solid, 29% yield. HPLC: Rt = 4.47 min, 98.2%. mp: 155.9–157.4°C. IR (KBr, cm^{-1}): 3318, 1582, 1523, 1490, 1453, 1423, 1316, 1294, 1253, 1224, 1146, 1120. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.51 (s, 3H), 4.20 (s, 2H), 6.62 (d, 2H, J = 8.8 Hz), 6.69 (d, 1H, J = 9.6 Hz), 7.14 (t, 3H, J = 6.0 Hz), 7.30 (s, 1H), 7.43 (dd, 1H, J = 2.4, 9.6 Hz), 7.46 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.0, 44.1, 111.7 (2C), 114.3–114.5 (F-Ar), 115.7–115.9 (F-Ar), 116.3, 122.2–122.3 (F-Ar), 128.8, 130.6 (2C), 130.7–130.8 (F-Ar), 135.5, 140.1–140.2 (F-Ar), 142.0, 151.4, 161.3, 161.6–163.8 (F-Ar), 196.2. MS *m/z*: 336 $[M]^+$.

*1-(3-Fluorophenyl)-5-((p-tolylamino)methyl)pyridin-2(1H)-one (**11**)*

White solid, 28% yield. HPLC: Rt = 14.21 min, 99.4%. mp: 135.1–136.5°C. IR (KBr, cm^{-1}): 3339, 1654, 1603, 1572, 1541, 1424, 1357, 1285, 1244, 1183, 1163. ^{1}H NMR (400 MHz, DMSO): δ 2.13 (s, 3H), 4.00 (d, 2H, J = 6.0 Hz), 5.85 (t, 1H, J = 6.0 Hz), 6.49 (d, 1H, J = 9.6 Hz), 6.54 (d, 2H, J = 6.0 Hz), 6.88 (d, 2H, J = 6.0 Hz), 7.24 (dd, 1H, J = 0.8 Hz, 8.0 Hz), 7.31 (d, 1H, J = 8.8 Hz), 7.35 (d, 1H, J = 9.6 Hz), 7.54 (dd, 1H, J = 2.4 Hz, 8.8 Hz), 7.58 (d, 1H, J = 6.0 Hz), 7.65 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.4, 43.4, 113.0 (2C), 114.6–114.8 (F-Ar), 115.3–115.5 (F-Ar), 120.8, 123.2 (F-Ar), 124.8, 129.6 (2C), 131.0–131.1 (F-Ar), 136.5, 141.7, 142.4–142.5 (F-Ar), 146.3, 160.9, 161.0–163.3 (F-Ar). MS *m/z*: 308 $[M]^+$.

*1-(3-Fluorophenyl)-5-((4-methoxyphenylamino)methyl)-pyridin-2(1H)-one (**12**)*

Hoary solid, 27% yield. HPLC: Rt = 6.79 min, 99.4%. mp: 126.7–127.9°C. IR (KBr, cm^{-1}): 3311, 3073, 3051, 3007, 2940, 2918, 2829, 1667, 1600, 1587, 1538, 1512, 1486, 1445, 1294, 1242, 1253, 1175, 1150, 1131. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.62 (s, 3H), 3.98 (d, 2H, J = 6.0 Hz), 5.65 (t, 1H, J = 6.0 Hz), 6.50 (d, 1H, J = 6.0 Hz), 6.58 (d, 2H, J = 8.8 Hz), 6.70 (d, 2H, J = 8.8 Hz), 7.24 (d, 1H, J = 8.0 Hz), 7.30 (d, 1H, J = 8.4 Hz), 7.35 (d, 2H, J = 10 Hz), 7.55 (d, 1H, J = 8.4 Hz), 7.58 (d, 2H, J = 10 Hz), 7.65 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 43.6, 55.2, 113.6 (2C), 114.2–114.5 (F-Ar), 114.5 (2C), 115.0–115.2 (F-Ar), 117.4, 120.4, 122.9 (F-Ar), 130.7–130.8 (F-Ar), 136.1, 141.4, 142.1–142.2 (F-Ar), 142.4, 150.9, 160.6, 160.6–163.0 (F-Ar). MS *m/z*: 324 $[M]^+$.

*1-(3-Fluorophenyl)-5-((m-tolylamino)methyl)pyridin-2(1H)-one (**13**)*

White solid, 30% yield. HPLC: Rt = 9.64 min, 98.5%. mp: 113.6–115.2°C. IR (KBr, cm^{-1}): 3348, 3073, 3066, 2918, 2822, 1674, 1600, 1578, 1515, 1482, 1453, 1427, 1320, 1297, 1283, 1257, 1227, 1164, 1150, 1131, 1116. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.17 (s, 3H), 4.02 (d, 2H, J = 6.0 Hz), 5.96 (t, 1H, J = 6.0 Hz), 6.37 (d, 1H, J = 7.2 Hz), 6.44 (m, 2H), 6.50 (d, 1H, J = 9.6 Hz), 6.95 (t, 1H, J = 8.0 Hz), 7.24 (d, 1H, J = 8.8 Hz), 7.33 (m, 2H), 7.56 (m, 2H), 7.66 (d, 1H, J = 2.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.4, 42.8, 109.8, 113.3, 114.3–114.5 (F-Ar), 115.1–115.3 (F-Ar), 117.2, 117.4, 120.5, 122.9–123.0 (F-Ar), 128.8, 130.8–130.9 (F-Ar), 136.2, 137.9, 141.5, 142.1–142.2 (F-Ar), 148.3, 160.7, 160.7–163.1 (F-Ar). MS *m/z*: 308 $[M]^+$.

*1-(3-Fluorophenyl)-5-((o-tolylamino)methyl)pyridin-2(1H)-one (**14**)*

White solid, 29% yield. HPLC: Rt = 9.74 min, 99.4%. mp: 94.7–96.1°C. IR (KBr, cm^{-1}): 3370, 3073, 2999, 2962, 2866, 2362, 1682, 1612, 1597, 1575, 1538, 1515, 1482, 1449, 1309, 1283, 1249, 1227, 1142, 1127. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.11 (s, 3H), 4.11 (d, 2H, J = 6.0 Hz), 5.43 (t, 1H, J = 6.0 Hz), 6.50 (m, 2H), 6.58 (d, 1H, J = 8.0 Hz), 6.96 (d, 1H, J = 7.2 Hz), 6.98 (d, 1H, J = 8.0 Hz), 7.24 (d, 1H, J = 8.8 Hz), 7.30 (dd, 1H, J = 2.4 Hz, 8.4 Hz), 7.36 (dt, 1H, J = 2.0 Hz, 10.0 Hz), 7.54 (d, 1H, J = 8.0 Hz), 7.63 (dt, 1H, J = 2.4 Hz, 8.4 Hz), 7.68 (d, 1H, J = 1.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.8, 42.7, 109.8, 114.3–114.5 (F-Ar), 115.0–115.2 (F-Ar), 116.1, 117.4, 120.4, 122.0, 122.9 (F-Ar), 126.7, 129.5, 130.7–130.8 (F-Ar), 136.2, 141.2, 142.1–142.2 (F-Ar), 145.8, 160.6, 160.6–163.0 (F-Ar). MS *m/z*: 308 $[M]^+$.

*5-((4-Chlorophenylamino)methyl)-1-(3-fluorophenyl)-pyridin-2(1H)-one (**15**)*

Slight sage green solid, 42% yield. HPLC: Rt = 11.39 min, 99.2%. mp: 130.9–132.1°C. IR (KBr, cm^{-1}): 3303, 3165, 3083, 3032, 2837, 1669, 1598, 1582, 1536, 1526, 1490, 1460, 1429, 1398, 1316, 1291, 1244, 1224, 1173, 1127. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.02 (d, 2H, J = 6.0 Hz), 6.28 (t, 1H, J = 6.0 Hz), 6.51 (d, 1H, J = 9.2 Hz), 6.64 (d, 2H, J = 9.2 Hz), 7.09 (d, 2H, J = 8.8 Hz), 7.25 (dd, 1H, J = 0.8 Hz, 8.0 Hz), 7.31 (dd, 1H, J = 2.4 Hz, 8.4 Hz), 7.36 (dt, 1H, J = 2.4 Hz, 10.0 Hz), 7.59 (d, 1H, J = 8.4 Hz), 7.69 (d, 1H, J = 2.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.8, 113.8 (2C), 114.3–114.5 (F-Ar), 115.0–115.2 (F-Ar), 116.7, 119.4, 120.6, 122.9 (F-Ar), 128.6 (2C), 130.7–130.8 (F-Ar), 136.3, 141.3, 142.0–142.1 (F-Ar), 147.2, 160.6, 160.6–163.0 (F-Ar). MS *m/z*: 328 $[M]^+$.

*1-Phenyl-5-((o-tolylamino)methyl)pyridin-2(1H)-one (**16**)*

White solid, 38% yield. HPLC: Rt = 8.49 min, 96.8%. mp: 121.9–122.6°C. IR (KBr, cm^{-1}): 3362, 3036, 2896, 1741, 1663, 1604, 1582, 1527, 1490, 1453, 1382, 1323, 1279, 1260, 1201, 1127. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.10 (s, 3H), 4.12 (d, 2H, J = 6.0 Hz), 5.38 (s, 1H), 6.48 (m, 2H), 6.57 (d, 1H, J = 7.6 Hz), 6.97 (br, 2H), 7.49 (m, 7H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.7, 42.8, 109.8, 116.0, 117.1, 120.4, 122.0, 126.6 (3C), 128.0, 129.1, 129.8 (2C), 136.4, 140.9, 141.0, 145.8, 160.8. MS *m/z*: 290 $[M]^+$.

*1-Phenyl-5-((p-tolylamino)methyl)pyridin-2(1H)-one (**17**)*

White solid, 41% yield. HPLC: Rt = 8.42 min, 98.5%. mp: 132.4–133.9°C. IR (KBr, cm^{-1}): 3318, 3044, 2999, 2918, 2844, 1671, 1608, 1582, 1519, 1493, 1453, 1430, 1379, 1316, 1275, 1249, 1205, 1124. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.13 (s, 3H), 4.00 (d, 2H, J = 6.4 Hz), 5.86 (t, 1H, J = 6.0 Hz), 6.48 (d, 1H, J = 9.2 Hz), 6.54 (d, 2H, J = 8.4 Hz), 6.88 (d, 2H, J = 8.0 Hz), 7.36 (d, 2H, J = 7.2 Hz), 7.45 (t, 1H, J = 7.2 Hz), 7.52 (m, 3H), 7.62 (d, 1H, J = 1.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.4, 43.4, 113.0 (2C), 117.5, 120.7 (2C), 124.8, 127.0, 128.4, 129.4 (2C), 129.7 (2C), 136.8, 141.2, 141.6, 146.3, 161.1. MS *m/z*: 290 $[M]^+$.

*5-((4-Methoxyphenylamino)methyl)-1-phenylpyridin-2-(1H)-one (**18**)*

Hoary solid, 27% yield. HPLC: Rt = 5.90 min, 98.7%. mp: 138.4–139.7°C. IR (KBr, cm^{-1}): 3296, 2992, 2836, 1667, 1600, 1582, 1530, 1515, 1453, 1401, 1297, 1275, 1249, 1235, 1179. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.62 (s, 3H), 3.98 (s, 2H), 5.65 (br, 1H), 6.48 (d, 1H, J = 9.6 Hz), 6.58 (d, 2H, J = 8.8 Hz), 6.71 (d, 2H, J = 8.8 Hz), 7.37 (d, 2H, J = 7.6 Hz), 7.44 (t, 1H, J = 7.6 Hz), 7.52 (m, 3H), 7.61 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 43.6, 55.2, 113.6 (2C), 114.5 (2C), 117.2, 126.6 (2C), 128.0, 129.0 (2C), 136.4, 140.9, 141.2, 142.4, 150.9, 160.8. MS *m/z*: 306 $[M]^+$.

*5-((4-Acetylphenylamino)methyl)-1-phenylpyridin-2(1H)-one (**19**)*

Yellow solid, 24% yield. HPLC: Rt = 4.45 min, 98.2%. mp: 166.5–168.8°C. IR (KBr, cm^{-1}): 3318, 1664, 1588, 1536, 1495, 1454, 1357, 1321, 1270, 1173, 1116. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H), 4.14 (d, 2H, J = 5.6 Hz), 6.50 (d, 1H, J = 9.6 Hz), 6.67 (d, 2H, J = 8.8 Hz), 6.96 (t, 1H, J = 6.0 Hz), 7.38 (d, 2H, J = 8.8 Hz), 7.44 (t, 1H, J = 7.2 Hz), 7.52 (m, 3H), 7.68 (d, 1H, J = 2.4 Hz), 7.72 (d, 2H, J = 8.8 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 26.1, 42.4, 111.4 (2C), 116.6, 120.7 (2C), 125.5, 126.8 (2C), 128.4, 129.3 (2C), 130.6 (2C), 136.9, 140.9, 141.3, 152.6, 161.1, 195.6. MS *m/z*: 318 $[M]^+$.

*1-Phenyl-5-((phenylamino)methyl)pyridin-2(1H)-one (**20**)*

White solid, 45% yield. HPLC: Rt = 8.22 min, 98.7%. mp: 138.2–139.2°C. IR (KBr, cm^{-1}): 3303, 3059, 3022, 2925, 1667, 1600, 1578, 1534, 1519, 1497, 1453, 1279, 1257, 1179, 1157, 1131. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.02 (d, 2H, J = 6.0), 6.06 (t, 1H, J = 6.0), 6.49 (d, 1H, J = 9.2 Hz), 6.54 (t, 1H, J = 7.6 Hz), 6.62 (d, 2H, J = 7.6 Hz), 7.06 (t, 2H, J = 8.0 Hz), 7.37 (d, 2H, J = 8.0 Hz), 7.44 (m, 2H), 7.53 (m, 3H), 7.64 (d, 1H, J = 2.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.8, 112.5 (2C), 116.1, 117.0, 120.4, 126.6 (2C), 128.1, 128.9 (2C), 129.1 (2C), 136.5, 140.9, 141.2, 148.3, 160.8. MS *m/z*: 276 $[M]^+$.

*5-((4-Chlorophenylamino)methyl)-1-phenylpyridin-2(1H)-one (**21**)*

Slight sage green solid, 38% yield. HPLC: Rt = 9.91 min, 99.0%. mp: 135.6–137.4°C. IR (KBr, cm^{-1}): 3296, 1660, 1597, 1575, 1530, 1515, 1486, 1449, 1397, 1382, 1312, 1279, 1260, 1172, 1124. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.02 (d, 2H, J = 6.0 Hz), 6.22 (d, 1H, J = 6.0 Hz), 6.48 (d, 1H, J = 9.6 Hz), 6.63 (m, 2H), 7.08 (m, 2H), 7.37 (m, 2H), 7.44 (m, 1H), 7.52 (dt, 3H, J = 6.4 Hz, 2.0 Hz), 7.63 (d, 1H, J = 2.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.8, 113.8 (2C), 116.5, 119.3, 120.5, 126.6 (2C), 128.1, 128.6 (2C), 129.1 (2C), 136.6, 140.9, 141.1, 147.2, 160.8. MS *m/z*: 310 $[M]^+$.

*1-Phenyl-5-((m-tolylamino)methyl)pyridin-2(1H)-one (**22**)*

White solid, 29% yield. HPLC: Rt = 8.44 min, 98.9%. mp: 118.5–119.0°C. IR (KBr, cm^{-1}): 3303, 1680, 1598, 1577, 1536, 1490, 1449, 1403, 1280, 1260, 1163, 1132. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.17 (s, 3H), 4.01 (d, 2H, J = 6.0 Hz), 5.96 (t, 1H, J = 6.0 Hz), 6.94 (t, 1H, J = 8.0 Hz), 7.36 (d, 2H, J = 8.0 Hz), 7.44 (t, 1H, J = 7, 6 Hz), 7.51 (d, 2H, J = 7.6 Hz), 7.54 (d, 1H, J = 2.8 Hz), 7.63 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.3, 42.8, 109.7, 113.2, 117.0, 117.0, 120.4, 126.6 (2C), 128.0, 128.7, 129.1 (2C), 136.5, 137.8, 140.9, 141.2, 148.3, 160.8. MS *m/z*: 290 $[M]^+$.

*5-((4-Chlorophenylamino)methyl)-1-(4-methoxyphenyl)-pyridin-2(1H)-one (**23**)*

White solid, 35% yield. HPLC: Rt = 21.03 min, 99.2%. mp: 151.0–151.8°C. IR (KBr, cm^{-1}): 3288, 3016, 2955, 2837, 1669, 1598, 1582, 1506, 1485, 1460, 1398, 1311, 1244, 1173, 1111. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.80 (s, 3H), 4.01 (d, 2H, J = 5.6 Hz), 6.27 (t, 1H, J = 5.6 Hz), 6.47 (d, 1H, J = 9.2 Hz), 6.63 (d, 2H, J = 8.8 Hz), 7.04 (d, 1H, J = 8.8 Hz), 7.09 (d, 1H, J = 8.8 Hz), 29 (d, 1H, J = 8.8 Hz), 7.50 (dd, 1H, J = 2.4 Hz, 9.2 Hz), 7.60 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.8, 55.4, 113.8 (2C), 114.2 (2C), 116.3, 119.3, 120.4, 127.7 (2C), 128.6 (2C), 133.7, 137.0, 141.0, 147.2, 158.7, 161.0. MS *m/z*: 340 $[M]^+$.

*5-((4-Acetylphenylamino)methyl)-1-(4-methoxyphenyl)-pyridin-2(1H)-one (**24**)*

Slight yellow solid, 33% yield. HPLC: Rt = 4.99 min, 98.9%. mp: 119.4–120.7°C. IR (KBr, cm^{-1}): 3272, 2965, 2832, 1664, 1593, 1577, 1531, 1511, 1454, 1357, 1270, 1250, 1173. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H), 3.80 (s, 3H), 4.12 (d, 2H, J = 5.6 Hz), 6.48 (d, 1H, J = 9.6 Hz), 6.66 (d, 2H, J = 8.8 Hz), 6.20 (t, 1H, J = 5.6 Hz), 7.05 (d, 2H, J = 8.8 Hz), 7.29 (d, 2H, J = 8.8 Hz), 7.50 (dd, 1H, J = 9.6 Hz, 2.8 Hz), 7.66 (d, 1H, J = 2.0 Hz), 7.72 (d, 2H, J = 8.8 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.8, 42.2, 55.4, 111.2 (2C), 114.1 (2C), 115.9, 120.4, 125.3, 127.7 (2C), 130.3 (2C), 133.6, 137.0, 140.8, 152.3, 158.7, 160.9, 195.0. MS *m/z*: 349 $[M+H]^+$.

*1-(4-Methoxyphenyl)-5-((4-methoxyphenylamino)methyl)-pyridin-2(1H)-one (**25**)*

White solid, 31% yield. HPLC: Rt = 6.76 min, 95.8%. mp: 169.0–170.3°C. IR (KBr, cm^{-1}): 3288, 3098, 3016, 2945, 2899, 2832, 1669, 1598, 1577, 1511, 1454, 1449, 1393, 1306, 1244, 1178, 1106. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.62 (s, 3H), 3.80 (s, 3H), 3.96 (d, 2H, J = 5.6), 5.64 (t, 1H, J = 5.6 Hz), 6.46 (d, 1H, J = 9.2 Hz), 6.58 (dd, 2H, J = 7.2 Hz, 2.4 Hz), 6.71 (d, 2H, J = 9.2 Hz), 7.04 (dd, 2H, J = 6.8 Hz, 2.0 Hz), 7.28 (dd, 2H, J = 6.8 Hz, 2.0 Hz), 7.52 (dd, 1H,

J = 9.6 Hz, 2.4 Hz), 7.57 (d, 1H, J = 2.0 Hz).^{13}C NMR (100 MHz, DMSO-d_6): δ 43.7, 55.2, 55.4, 113.6 (2C), 114.2 (2C), 114.6 (2C), 117.0, 120.2, 127.7 (2C), 133.8, 136.8, 141.1, 142.5, 150.9, 158.7, 161.0. MS m/z: 336 $[M]^+$.

*1-(4-Methoxyphenyl)-5-((phenylamino)methyl)pyridin-2-(1H)-one (**26**)*

White solid, 32% yield. HPLC: Rt = 6.91 min, 99.2%. mp: 139.7–140.5°C. IR (KBr, cm^{-1}): 3375, 3298, 3108, 3011, 2934, 2837, 2356, 2340, 1664, 1603, 1582, 1541, 1506, 1454, 1306, 1244, 1173, 1106. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.80 (s, 3H), 4.01 (d, 2H, J = 6.0 Hz), 6.05 (t, 1H, J = 6.0 Hz), 6.46 (d, 1H, J = 9.2 Hz), 6.54 (t, 1H, J = 7.2 Hz), 6.61 (d, 2H, J = 8.0 Hz), 7.04 (d, 2H, J = 8.8 Hz), 7.07 (d, 2H, J = 8.0 Hz), 7.28 (d, 2H, J = 8.8 Hz), 7.52 (dd, 1H, J = 2.0 Hz, 9.2 Hz), 7.59 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.8, 55.4, 112.5 (2C), 114.2 (2C), 116.1, 116.8, 120.3, 127.7 (2C), 128.9 (2C), 133.8, 136.7, 141.0, 148.3, 150.9, 158.7, 161.0. MS m/z: 306 $[M]^+$.

*1-(4-Methoxyphenyl)-5-((p-tolylamino)methyl)pyridin-2-(1H)-one (**27**)*

White solid, 41% yield. HPLC: Rt = 10.93 min, 99.0%. mp: 155.0–156.1°C. IR (KBr, cm^{-1}): 3288, 3016, 2945, 2904, 2832, 1675, 1598, 1588, 1516, 1506, 1460, 1403, 1296, 1250, 1178, 1101. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.12 (s, 3H), 3.79 (s, 3H), 3.98 (d, 2H, J = 6.0 Hz), 5.86 (t, 1H, J = 6.0 Hz), 6.43 (d, 1H, J = 9.2 Hz), 6.52 (d, 2H, J = 8.4 Hz), 6.87 (d, 2H, J = 8.0 Hz), 7.03 (d, 2H, J = 8.8 Hz), 7.26 (d, 2H, J = 8.8 Hz), 7.50 (dd, 1H, J = 9.6 Hz, 2.4 Hz), 7.54 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.0, 43.1, 55.4, 112.6 (2C), 114.1 (2C), 116.8, 120.2, 124.4, 127.6 (2C), 129.2 (2C), 133.7, 136.6, 140.9, 146.0, 150.9, 158.6, 160.9. MS m/z: 320 $[M]^+$.

*1-(4-Methoxyphenyl)-5-((o-tolylamino)methyl)pyridin-2-(1H)-one (**28**)*

White solid, 36% yield. HPLC: Rt = 6.94 min, 94.8%. mp: 130.6–132.3°C. IR (KBr, cm^{-1}): 3318, 3032, 3006, 2965, 2929, 2837, 1664, 1603, 1582, 1506, 1454, 1398, 1311, 1244, 1168, 1132. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.10 (s, 3H), 3.79 (s, 3H), 4.10 (d, 2H, J = 6.0 Hz), 5.43 (t, 1H, J = 6.0 Hz), 6.45 (d, 1H, J = 8.8 Hz), 6.50 (t, 1H, J = 7.6 Hz), 6.57 (d, 1H, J = 7.6 Hz), 6.96 (dd, 2H, J = 7.8 Hz, 2.8 Hz), 7.03 (dd, 2H, J = 6.8 Hz, 2.4 Hz), 7.27 (dd, 2H, J = 6.8 Hz, 2.4 Hz), 7.55 (dd, 1H, J = 9.2 Hz, 2.4 Hz), 7.59 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.8, 42.8, 55.4, 109.8, 114.1 (2C), 116.0, 116.9, 120.3, 122.0, 126.6, 127.7 (2C), 129.8, 133.8, 136.7, 140.8, 145.8, 158.7, 161.0. MS m/z: 320 $[M]^+$.

*1-(4-Methoxyphenyl)-5-((m-tolylamino)methyl)pyridin-2-(1H)-one (**29**)*

White solid, 38% yield. HPLC: Rt = 6.71 min, 98.0%. mp: 104.1–105.3°C. IR (KBr, cm^{-1}): 3318, 3006, 2919, 2832, 1664, 1597, 1582, 1536, 1506, 1460, 1429, 1393, 1250, 1188, 1135, 1116. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.17 (s, 3H), 3.80 (s, 3H), 4.00 (d, 2H, J = 5.6 Hz), 5.95 (t, 1H, J = 6.0 Hz), 6.36 (d, 1H, J = 7.6 Hz), 6.41 (d, 1H, J = 8.0 Hz), 6.46 (d, 2H, J = 9.6 Hz), 6.94 (t, 1H, J = 8.0 Hz), 7.04 (dd, 2H, J = 9.6 Hz, 2.0 Hz), 7.28 (dd, 2H, J = 6.8 Hz, 2.4 Hz), 7.46 (dd, 2H, J = 9.2 Hz, 2.8 Hz), 7.58 (d, 1H, J = 1.6 Hz). ^{13}C NMR (400 MHz, DMSO-d_6): δ 21.3, 42.8, 55.4, 109.7, 113.2, 114.2 (2C), 116.8, 117.0, 120.3, 127.7 (2C), 128.7, 133.8, 136.8, 137.8, 141.0, 148.3, 158.7, 161.0. MS m/z: 320 $[M]^+$.

General procedure for the synthesis of C-5 ethers derivatives (30–32)

*Synthesis of 1-phenyl-5-(m-tolyloxymethyl)pyridin-2(1H)-one (**30**)*

0.1 mol *m*-cresol was heated at reflux in 40 mL 10.5% NaOH for 1 h, then the water was removed to obtain white salt. To the salt in 30 mL CH_3CN was added **7b** in 30 mL CH_3CN, stirred and refluxed 2.5 h. The mixture was filtered, the filtrate was evaporated to dryness and **30** was obtained upon column chromatography (EtOAc/petroleum ether).

White solid, 47% yield. HPLC: Rt = 24.43 min, 95.5%. mp: 93.4–95.0°C. IR (KBr, cm^{-1}): 3032, 2909, 2888, 1680, 1623, 1577, 1541, 1485, 1449, 1393, 1367, 1296, 1260, 1152, 1127. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.27 (s, 3H), 4.83 (s, 2H), 6.54 (d, 2H, J = 9.2 Hz), 6.80 (m, 3H), 7.17 (t, 1H), 7.40 (d, 2H, J = 7.6 Hz), 7.45 (d, 2H, J = 6.8 Hz), 7.52 (t, 2H, J = 7.2 Hz), 7.59 (d, 1H, J = 9.2 Hz), 7.82 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.1, 66.0, 111.8, 114.5, 115.5, 120.6, 121.6, 126.8 (2C), 128.3, 129.1 (2C), 129.3, 138.4, 139.0, 140.7, 141.4, 158.2, 160.9. MS m/z: 291 $[M]^+$.

*5-((4-Chlorophenoxy)methyl)-1-phenylpyridin-2(1H)-one (**31**)*

White solid, 44% yield. HPLC: Rt = 9.44 min, 99.1%. mp: 112.9–114.7°C. IR (KBr, cm^{-1}): 3129, 1675, 1613, 1588, 1526, 1485, 1388, 1291, 1234, 1173, 1132. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.86 (s, 2H), 6.53 (d, 1H, J = 9.6 Hz), 7.04 (d, 2H, J = 8.8 Hz), 7.34 (d, 2H, J = 8.4 Hz), 7.42 (d, 2H, J = 7.6 Hz), 7.46 (t, 1H), 7.51–7.55 (t, 2H), 7.62 (dd, 1H, J = 9.6 Hz, 2.4 Hz), 7.86 (d, 1H, J = 2.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 66.5, 114.0, 116.6 (2C), 120.6, 124.5, 126.8 (2C), 128.2, 129.1 (2C), 129.2 (2C), 138.8, 140.6, 141.4, 157.0, 160.8. MS m/z: 311 $[M]^+$.

*5-(Phenoxymethyl)-1-phenylpyridin-2(1H)-one (**32**)*

White solid, 42% yield. HPLC: Rt = 6.15 min, 99.6%. mp: 109.6–110.4°C. IR (KBr, cm^{-1}): 3021, 1685, 1613, 1598, 1526, 1501, 1454, 1398, 1357, 1229, 1168, 1137. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.86 (s, 2H), 6.54 (d, 1H, J = 9.6 Hz), 6.95 (t, 1H), 7.01 (d, 2H, J = 7.6 Hz), 7.30 (t, 2H), 7.42 (d, 2H, J = 8.4 Hz), 7.46 (m, 1H), 7.52 (t, 2H), 7.62 (dd, 1H, J = 8.8 Hz, 2.4 Hz), 7.86 (d, 1H, J = 2.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 66.0, 114.4, 114.8 (2C), 120.6, 120.8, 126.7 (2C), 128.2, 129.1, 129.5, 138.5, 140.6, 141.4, 158.1, 160.8. MS m/z: 277 $[M]^+$.

Pharmacophore modeling

The structures of compound **5a** and **9** were built by MOE. The hydrogens and partial charges of the compounds were preliminarily adjusted under forcefield MMFF94x. The compounds were flexibly aligned and the pharmacophore features were created by "Pharmacophore Query Editors" modules for the best alignment with default parameters.

Biological assays

Materials

The cisplatin and L-mimosine were purchased from Sigma. Antibodies against β-actin were from Abcam (Cambridge, MA, USA). Cell culture media and reagents were obtained from Hyclone. All other reagents were of molecular biology grade from Sigma (St Louis, MO, USA) or Invitrogen (Carlsbad, CA, USA).

Table 4. Primers and annealing temperature used for real-time PCR.

Gene	Primer sequence	Annealing temperature
Human β-actin	F: 5′-TGGCACCCAGCACAATGAA-3′ R: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′	60°C
eIF3a	F: 5′-TCAAGTCGCCGGGACGATA-3′ R: 5′-CCTGTCATCAGCACGTCTCCA-3′	60°C

Cell viability assay

Cell proliferation assay was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit, according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, A549 or NIH3T3 cells were seeded in 96-well plates and allowed to grow for 24 h followed by incubation with compounds for 24 h. Culture medium was then removed. Pipet 20 μL of MTS solution into each well of the 96-well assay plate containing the samples in 100 μL of culture medium. Incubate the plate in a humidified, 5% CO_2 atmosphere for 2 h at 37°C. Record the absorbance at 490 nm using a Multiskan Ascent 354 microplate reader (Thermo Labsystems, Helsinki, Finland). The absorption value was determined by Ascent Software™ (Thermo Labsystems, Helsinki, Finland) and IC_{50} values were obtained from the dose–response curves using GraphPad Prism™ 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Immunoblot analysis assay

A549 cells (1×10^5/well) were treated with compound **5a**, **9**, **22**, **30**, L-mimosine, PFD or AKF-PD for 8 h. After protein sample preparation, expression of eIF3a and β-actin were examined by Western blot analysis as previously described. Briefly, cells were lysed in radio immuno precipitation assay (RIPA) lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM sodium fluoride, 100 mg/mL phenylmethylsulfonylfluoride (PMSF), 100 mg/mL dithiothreitol (DTT)] at 4°C for 30 min. The lysates were clarified by centrifugation at 10,000 *g* for 10 min at 4°C; protein concentration of the lysate samples was determined using Bio-Rad Protein Assay kit. For Western blot analysis, protein samples were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were then blocked with 5% skimmed milk and incubated with primary antibodies (anti-eIF3a and anti-β-actin) overnight followed by washing. The reaction was detected by IRDye® 800CW, conjugated goat (polyclonal) anti-mouse or rabbit antibody (LI-COR Biosciences, Lincoln, NE, USA) and visualized by Odyssey Scanner (LI-COR Biosciences).

Real-time reverse transcriptase PCR

A549 cells (1×10^5/well) were treated with **22**, **30**, L-mimosine, PFD for 8 h. mRNA levels of eIF3a were analyzed by real-time RT x2010;PCR as described previously using specific primers and normalized to that of β-actin. Briefly, the cells were harvested and total RNA was isolated using Trizol reagent (Invitrogen). One microgram of total RNAs were reverse transcribed using Reverse Transcription System Kit (Promega) according to the manuscript's instructions. The real-time PCR were carried out in a Stratagene Mx3000p Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA) using SYBR GreenER Qpcr Supermix Universal Kit (Invitrogen) according to the manuscript's instructions. The complete sequences of the oligonucleotide primers, annealing temperature are given in Table 4. The C_t value was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold. The C_t value of each product was determined and normalized against that of internal control, β-actin.

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