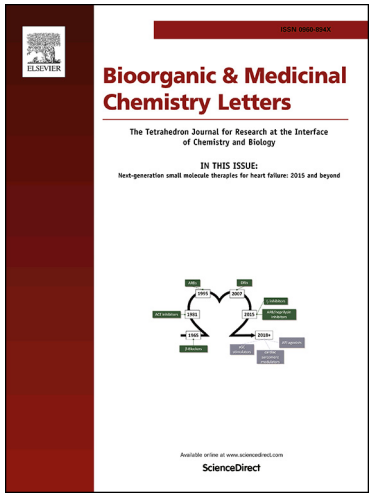
Accepted Manuscript



2-Pyridineformamide N(4)-ring incorporated thiosemicarbazones inhibit MCF-7 cells by inhibiting JNK pathway

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PII: S0960-894X(19)30250-1
DOI: https://doi.org/10.1016/j.bmcl.2019.04.031
Reference: BMCL 26398

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 26 February 2019
Revised Date: 8 April 2019
Accepted Date: 18 April 2019

Please cite this article as: Shakya, B., Shahi, N., Ahmad, F., Nath Yadav, P., Raj Pokharel, Y., 2-Pyridineformamide N(4)-ring incorporated thiosemicarbazones inhibit MCF-7 cells by inhibiting JNK pathway, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.04.031

2-Pyridineformamide N(4)-ring incorporated thiosemicarbazones inhibit MCF-7 cells by inhibiting JNK pathway

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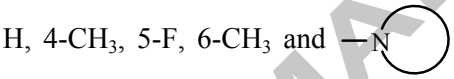
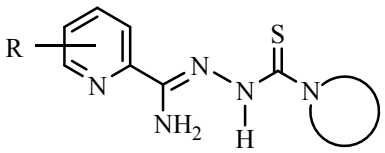
ABSTRACT

In an effort to develop a more potent anticancer therapeutic agent, a series of 2-pyridineformamide thiosemicarbazones (R = H, 4-CH_3, 5-F, 6-CH_3 and ) have been synthesized and evaluated for their anti-cancer activities against the cancer cells MCF-7 (breast cancer cell line), A-431and A375 (epidermoid carcinoma cell line), and HeLa (cervical cancer cell line) using MTT assay. All these 2-pyridineformamide thiosemicarbazones exhibited anti-proliferative activities towards these cell lines. **5FAmPyrr** possess most profound effects against MCF-7 cells with IC_{50} of 0.9 µM. In flow cytometry using Propidium Iodide, **5FAmPyrr** was found to induce cell death significantly in a dose dependent manner (100 nM to 3 µM) and inhibited colony formation of MCF-7 cells. This compound induced pro-apoptotic protein Bax and inhibited anti apoptotic protein Bcl-2 as well as both c-Jun and Jun N-terminal kinase (abbreviated as JNK) in concentration dependent manner. Further pro-caspase 3 and PARP were inhibited by **5FAmPyrr** at concentration of 3 µM. The results suggest that **5FAmPyrr** exhibit anticancer potency and induced cell death by inhibiting MAPK signaling and inducing intrinsic apoptotic pathway. All these indicate that 2-pyridineformamide thiosemicarbazones could be developed as future therapeutics agents to treat cancer.



Key words: A431, A375, cell viability, HeLa, MCF-7, 2-pyridineformamide thiosemicarbazone.

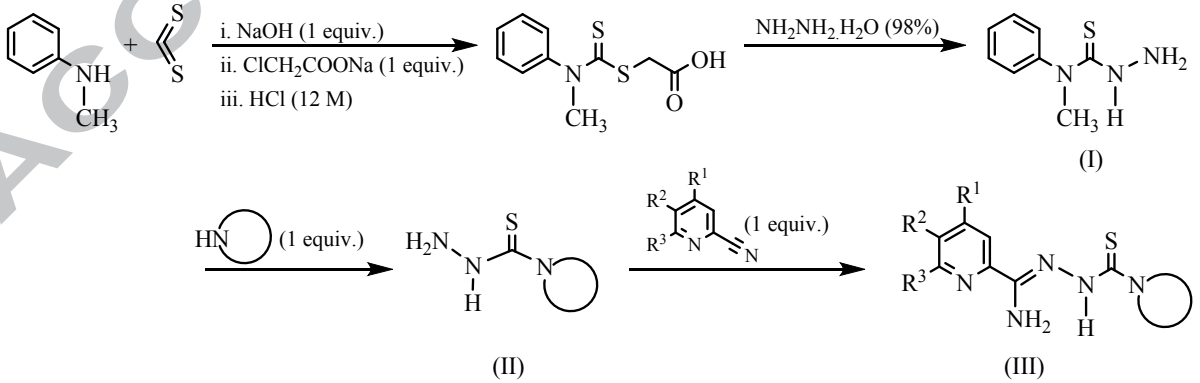
Thiosemicarbazones are important class of compounds with potential biological activities, such as antibacterial, antiviral, antimalarial and antitumor activities.[1-3] Anti-microbial activity of thiosemicarbazone was first recognized by Domagk in 1946.[4,5] Antiviral properties of thiosemicarbazone was discovered in sixties and detailed structure activity relationship study of this class of compound ultimately led to commercialization of methisazone, Marboran® for the treatment of

smallpox.[6] Anticancer activity of heterocyclic thiosemicarbazone (HCT) was first reported by Brockman et. al with pyridine-2-carboxaldehyde thiosemicarbazone which was found potent to prolong the life span of mice bearing the L1210 leukemia.[7] This observation encouraged the synthesis and anticancer screening of different derivatives of thiosemicarbazones with modification in the heterocyclic ring system, variations in the ring substituents and thiosemicarbazone side chain.[8-10]

Anticancer drugs exert their activity by inhibiting different biochemical process occurring in the cell cycle resulting in cell death. The most common target of many currently used chemotherapy drugs is inhibition of DNA or RNA synthesis and repair.[11-13]

Ribonucleotide reductase (RR) plays a central role for the generation of the cytosine, adenine, and guanine deoxyribonucleotide 5'-triphosphate building blocks of DNA synthesis and repair.[14] The α-(N)-heterocyclic thiosemicarbazones are one of the most potent inhibitor of RR activity which ultimately affect the DNA synthesis and prevents cancer cells from division.[15] A strong inhibitor of RR would be an effective anticancer agent. One of the member of thiosemicarbazone, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP), Triapine®, has been found as a potent inhibitor of the RR activity.[16] Currently, Triapine® has reached phase II clinical trial on several cancer types; in patients with recurrent or metastatic head and neck squamous cell carcinoma[17] and a multicenter phase II trial in combination with gemcitabine in advanced non-small-cell lung cancer[18]. Over expression of RR activity correlates to an increase in cell invasion potential. Over expression of RR has also been reported in human pancreatic adenocarcinoma, which is associated with the resistance to gemcitabine, a drug for the treatment of advanced pancreatic cancer.[19-20]

We recently discovered that 2-pyridineformamide thiosemicarbazones retard the tolerance to nutrition starvation of pancreatic cancer cell.[21] Pancreatic cancer is known to be the most fatal form of cancer having lowest 5 year survival rate of <5% known for all the cancers.[22] The result of our previous study encouraged us to explore further synthesis of 2-pyridineformamide thiosemicarbazone derivatives and evaluate their preferential cytotoxic activity against other cancers also. Therefore, we prepared more new 2-pyridineformamide thiosemicarbazone derivatives with variations in *N*(4)-substitution as potential anti-cancer candidates. The synthetic route is illustrated in Scheme 1. The common intermediate 4-methyl-4-phenyl-3-thiosemicarbazide (I) was first prepared according to the procedure described by Scovill.[23] Transamination of I with an amine gave the corresponding *N*- ring incorporated thiosemicarbazide (II) which was converted to thiosemicarbazone (III). The structures and yields of the 23 compounds synthesized are presented in Table 1. The structures of all the synthesized compounds were confirmed using NMR spectroscopic data and HRFABMS data.

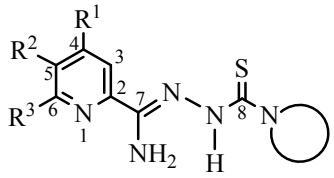


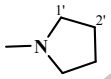
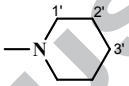
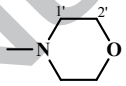
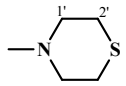
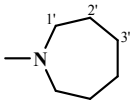
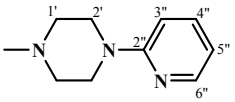
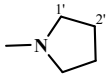
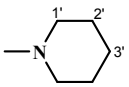
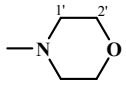
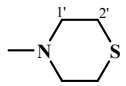
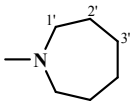
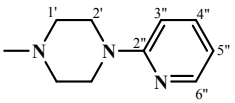
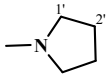
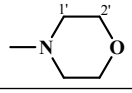
Scheme 1. Synthesis of *N*(4)-ring incorporated 2-pyridineformamide thiosemicarbazones

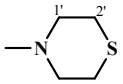
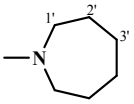
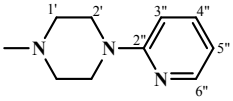
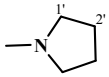
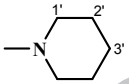
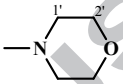
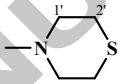
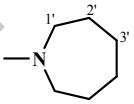
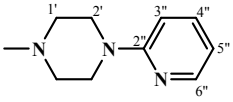
The synthesized compounds (Table 1) were screened for their anti-cancer potential in terms of viability of following cancer cell lines- MCF-7 (breast cancer cell line), A-431and A375 (epidermoid carcinoma cell line), HeLa (cervical cancer cell line) by MTT assay and percentage inhibition was calculated on

the basis of percentage viability (see supplementary data). The IC_{50} values determined are shown in Table 2.

Table 1. *N*(4)-Ring incorporated 2-Pyridineformamide thiosemicarbazones



Compound	R^1	R^2	R^3	—N	Yield (%)
1	H	H	H		46
2	H	H	H		21
3	H	H	H		60
4	H	H	H		59
5	H	H	H		24
6	H	H	H		45
7	CH_3	H	H		24
8	CH_3	H	H		29
9	CH_3	H	H		41
10	CH_3	H	H		32
11	CH_3	H	H		45
12	CH_3	H	H		63
13	H	F	H		45
14	H	F	H		50

15	H	F	H		52
16	H	F	H		40
17	H	F	H		61
18	H	H	CH_3		26
19	H	H	CH_3		21
20	H	H	CH_3		54
21	H	H	CH_3		41
22	H	H	CH_3		19
23	H	H	CH_3		42

Most of the synthesized compounds exhibited moderate to augmented effect against MCF-7 cell line except **4MeAmMorp**, **6MeAmTMorp** and **6MeAmPypz** with IC_{50} (0.9-74 µM). Likewise, all the compounds have good anti-proliferative activity against epidermoid carcinoma cell lines A-431 and A375 with IC_{50} (0.9-19.6 µM) and (0.6-14 µM) respectively. Synthesized compounds were also found to be inhibitory against HeLa cell line with IC_{50} between 0.9-38.5 µM.

Table 2. IC_{50} values of compounds against different cancer cell lines

Compound No.	Compound Name	(IC_{50}, µM)			
		MCF-7	A431	A375	HeLa
1	HAmPyrr	3.6	1.7	0.6	6.2
2	HAmPip	8.5	6.9	0.68	3.8
3	HAmMorp	4.7	4.8	0.68	7.3
4	HAmTmorp	8.8	6.7	0.76	7.8
5	HAmHexim	2.6	3	0.74	6
6	HAmPypz	13.7	4.3	0.7	8.6
7	4MeAmPyrr	1.2	0.9	0.72	1.9
8	4MeAmPip	18.8	18.9	1.9	7.7
9	4MeAmMorp	>100	6.8	1.6	38.5
10	4MeAmTmorp	6.9	7.2	1.2	15.5
11	4MeAmHexim	6.9	8	0.8	7
12	4MeAmPypz	12.3	15.5	0.78	10.5
13	5FAmPyrr	0.9	1	0.86	1.8
14	5FAmMorp	22.1	4	1.5	1.7
15	5FAmTmorp	21.4	1.2	0.9	1.7

16	5FAmHexim	21.1	1	0.96	0.9
17	5FAmPypz	22	13.4	1.4	2
18	6MeAmPyrr	61.8	4	2.9	2.7
19	6MeAmPip	74	12.4	1	2
20	6MeAmMorp	21.5	4.3	14	2.2
21	6MeAmTMorp	>100	2.6	1.4	1.8
22	6MeAmHexim	54.7	14.7	1.8	1
23	6MeAmPypz	>100	19.6	0.85	0.9

This primary screening result revealed that compound **5FAmPyrr** was most active against all the cell lines with IC_{50} values lying between 0.9-1.8 µM and among them exhibited profound anti-proliferative activity against MCF-7 cell line with IC_{50} of 0.9 µM. This most potent candidate was further chosen for detailed study, however lower concentrations (100 nM, 300 nM,1 µM and 3 µM) were chosen for subsequent studies. To determine the cancer cell specific selectivity of **5FAmPyrr**, crystal violet assay was performed for cell viability with normal human cell lines, HEK 293 and PNT2 (normal prostate epithelium cells), 3000 cells/well were incubated with **5FAmPyrr** in 96 well plates (control, vehicle control, 100nM, 300nM, 1µM and 3µM) for 72 hours. We observed that **5FAmPyrr** showed partial cytotoxic effects to non-cancerous cells but the percentage of cell death that was observed in cancer cells was significantly higher as compared with non-cancerous cells (figure 1C). We could conclude that 5FAmPyrr has potent anti-cancer effects and selectively target for cancer cells. To explore the reason behind this anti-proliferative activity of **5FAmPyrr,** cell death analysis by flow cytometry using Propidium Iodide was done and it was found that **5FAmPyrr** significantly induced cell death in a dose dependent manner as shown in figure 1 A. Cell Viability assay was performed in MCF-7 cells after the treatment of **5FAmPyrr** in 100 nM, 300 nM, 1 µM and 3 µM concentration for 72 hours. MTT assay results showed that **5FAmPyrr** inhibit MCF-7 cells growth in concentration dependent manner as shown in Figure 1B. Furthermore long term treatment of **5FAmPyrr** significantly inhibits clonogenicity of MCF7 cells in dose dependent manner, as shown in figure 2 A; 300 nM treated well showed the significant inhibition of both colony number and size respectively.

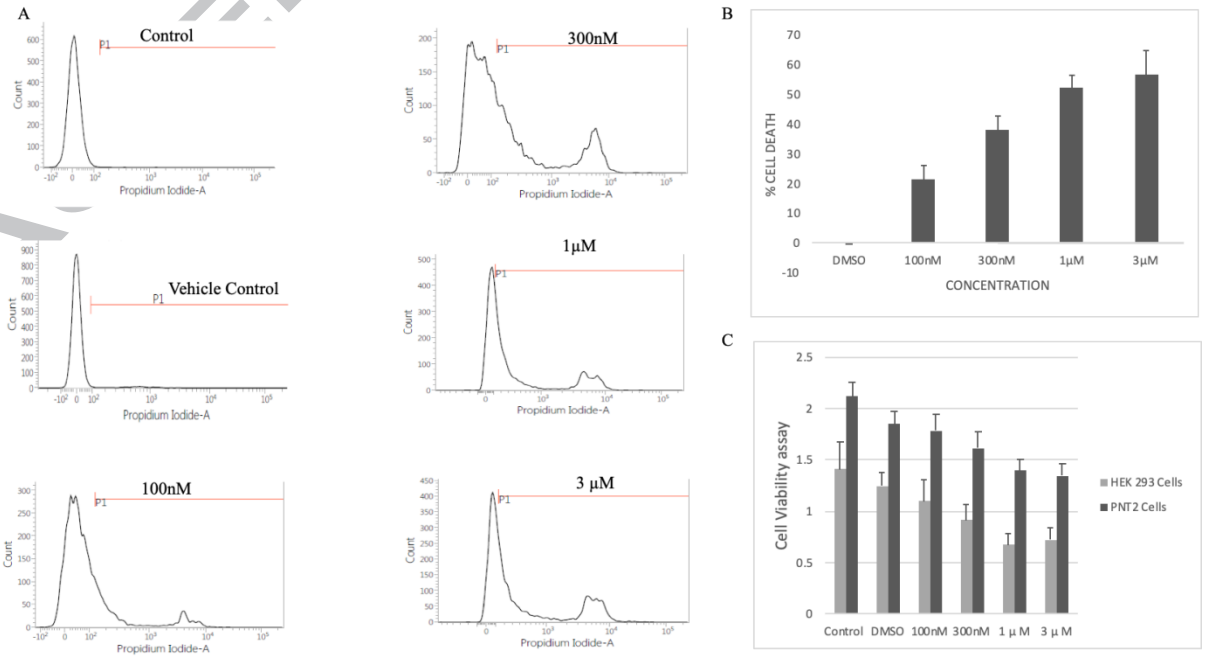


Figure 1: **Effect of 5FAmPyrr on cell survival in MCF-7 cells**. A &B. MCF-7 cells (30,000cells/well) were incubated at 37˚C with **5FAmPyrr** (control, vehicle control, 100nM, 300nM, 1µM and 3µM) for 48h. Following incubation, cells were harvested and stained with propidium iodide and analyzed by flow cytometry. Bar diagram showing % of cell death after treating with increasing concentrations (control, vehicle control, 100nM, 300nM, 1µM and 3µM). C. HEK 293 and PNT2 (normal prostate epithelium cells) (3000 cells/well) were incubated with **5FAmPyrr** in 96 well plates (control, vehicle control, 100nM, 300nM, 1µM and 3µM) for 72 hours and crystal violet assay (CVs) was performed to determine the cytotoxic effect.

Next to unravel the molecular machinery, participation of which led to significant cell death in MCF-7 cells, expression of Bcl-2 family members like pro-apoptotic Bax and anti-apoptotic Bcl-2 were analyzed by immunoblotting. It was found that pro-apoptotic Bax was equally upregulated in all concentration as shown in figure 2B, whereas anti-apoptotic Bcl-2 was equally down-regulated at all concentration of **5FAmPyrr**. This modulation of Bax and Bcl-2 following treatment of MCF-7 cells with **5FAmPyrr** committed cells to undergo cell death, as the ratio of Bax to Bcl-2 is a critical aspect for a cell to undergo apoptosis and then cell death.[24]

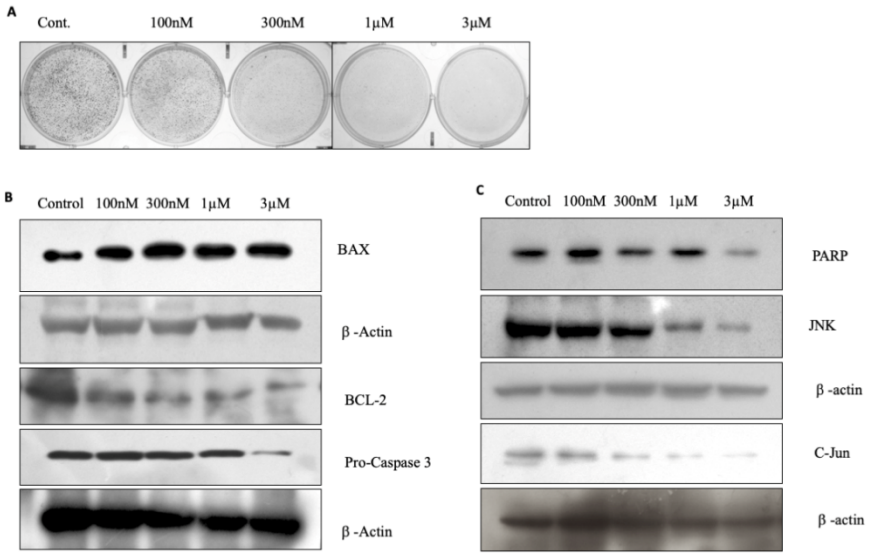


Figure 2: **Effect of 5FAmPyrr on expression of apoptotic proteins and mechanism of action.**
A. MCF-7 cells (1200/well) were treated with increasing concentration of **5FAmPyrr** (Control, 100nM, 300nM, 1µM and 3µM) for 21 days with every 72 hours treatment and after the colonies developed, they were fixed, stained with crystal violet solution. Colony formation assay showed that **5FAmPyrr** effectively inhibit colony formation in a concentration dependent manner.
B & C. **Expression of anti- and pro-apoptotic proteins following treatment with 5FAmPyrr.** Cell lysate was prepared after treating MCF-7 cells with **5FAmPyrr** (Control, 100nM, 300nM, 1µM and 3µM for 48 h). Protein lysate was resolved on SDS-PAGE and immunoblotting was performed using appropriate primary and secondary antibodies. Protein expression was studied for BAX, BCL2, Pro-Caspase-3, PARP, JNK, and C-Jun respectively and β-actin was used as loading control in each experiment.

We further checked the status of caspase 3 an executioner caspase, and Poly(ADP-ribose) polymerase (PARP) one of the substrate of caspase 3 and whose cleavage induces apoptosis in cancer cells.[25] Verily we found inhibition in pro casapase-3 and PARP-1 as shown in figure 2C, which suggest an intrinsic nature of cell death induction by **5FAmPyrr** against breast cancer MCF-7 cell lines. This activation of

cell death led us to check the expression of Jun N-terminal kinase (JNK), a critical and one of major member of MAPK super family whose involvement has been reported both in cell survival and apoptosis[26] and its hyperactivation is a common anomaly in a number of pathological conditions including cancer and a growing body of evidence suggests that impairing JNK signaling could be an important cancer therapeutic strategy.[27] Compound **5FAmPyrr** was found to be inhibiting JNK at 1 and 3 μM as shown in figure 2 which supports the apoptotic cell death mechanism followed by treatment of cells. Moreover c-Jun an important downstream target of JNK and multi-faceted protein possess both proliferative and apoptosis inducing activities[28] was also found to be inhibited in a dose dependent manner which further strengthen the finding that **5FAmPyrr** induces cell death hampering JNK/c-Jun proliferative signals.

Acknowledgments

This work was supported by a Grant from University Grants Commission of Government of Nepal to B.S. We thank Dr. Rajendra Shakya, Wayne State University, USA for IR and NMR data acquisition and Dr. Suresh Awale, University of Toyama, Japan for HR-FAB MS analysis. South Asian University for providing support to performed biological assays.

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29. *Cell culture and treatment*: MCF7, A431, A375 and HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 ^{o}C in 5% CO_2 incubator. Cells were treated with varying concentrations of a series of **23** synthetic 2-pyridineformamide thiosemicarbazones (1, 3, 10, 100 μM) in DMSO for 72 h for screening and followed by **5FAmPyrr** experimentation with (0.1, 0.3, 1, 3μM) concentration respectively.0.1% DMSO was used as vehicle control.
30. *MTT Assay (cell viability assay)*: The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell viability. MCF7, A431, A375 and HeLa cells as well as HEK 293 and PNT2 (normal prostate epithelial cells) were seeded in a 96 well plate at a density of 3000 cells/100μL/well. Following attachment of cells left overnight, cells were treated with varying concentrations (1-100 μM) of the series of 23 compounds as well as (100 nM-3 μM) **5FAmPyrr**in another 100 μL of media and were kept in incubation for 72 h. Afterwards, 20 μL of MTT (5 mg/mL in PBS) was added to each well and cells were incubated for another 3 h at 37 °C. The supernatant was removed carefully and 150 μL DMSO was added to each well to dissolve MTT formazan crystals. The plate was shaken on a rocker for 15-20 min and absorbance value of each well was determined using a multi-well plate reader (Biotek, Winooski, USA) at 570 nm. The wells treated with DMSO were taken as vehicle control respectively. Data were taken from the number of replicates and percentage viability was determined with respect to DMSO treated cells.
31. *Apoptosis and cell death assays*: For flow cytometry study MCF7 cells were cultured in a six-well culture plates, incubated with concentrations 100 nM - 3 μM of **5FAmPyrr** for 72 h. Cells were harvested, washed with PBS and incubated with Propidium Iodide (5μg/mL) for 20 min at 4 °C. Samples were acquired using FACS verse (BD Biosciences) for cell death using flow cytometry. Cells with DMSO treatment were taken as control.
32. *Western Blotting*: 1.25×10^5 MCF7 cells were seeded into each well of six well plates and exposed to different concentration of **5FAmPyrr** (0.1, 0.3, 1, 3, and 10 μM) for 72 h. Following incubation, cells were washed with cold PBS and lysed using 2XSDS lysis buffer (0.5 MTris-HCl, pH 6.8, glycerol, 10% (w/v) SDS). Cell lysates were sonicated once for 10 sec. at 30% amplitude. Protein estimation was done by BCA method and 20-25 μg protein samples were resolved on 12% polyacrylamide SDS gel and electrophoretically transferred to PVDF membranes. Membranes were then blocked with 5% BSA in TBST buffer for 1 h at room temperature, incubated with primary antibodies (1:500 and 1:1000) for overnight, and subsequently with the conjugated secondary antibody (1:5000) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (Bio-Rad).
33. *Colony Formation Assay*: MCF7 cells were seeded in six well plates at a density of 500 cells per well. Following adherence of cells left for overnight and next day cells were treated with **5FAmPyrr**. After every 72 h media was changed and cells were treated with **5FAmPyrr** and the

experiment lasts for 21 days. After the colony were fully grown, cells were washed with PBS, fixed with 3.7% formaldehyde for 20 min, washed with PBS and then stained using 0.4% crystal violet followed by rinsing the wells with PBS 4 to 5 times. Colonies were counted using Image J software.