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Synthesis of novel 5-arylidenethiazolidinones with apoptotic properties *via* a three component reaction using piperidine as a bifunctional reagent

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Abstract

The synthesis of a new library of 5-arylidenethiazolidinone compounds using an efficient three component reaction with thiazolidine-2, 4-dione, piperidine and appropriate aldehydes is reported. This reaction is excellently high yielding, tolerant towards a variety of aldehydes and provides access to these compounds in single step (in comparison to low yielding multistep syntheses reported in the literature). Once the reaction is complete, the desired product precipitates out of the reaction mixture and are isolated by filtration and purified by washing and recrystallization. These compounds revealed antiproliferative activities against human breast cancer cells (MCF7 and MDA). Phenotypic profiling established the most active compound **17i** (EC_{50} = 4.52 μM) as an apoptotic agent. A novel chemical proteomics approach identified β-actin-like protein 2, γ-enolase and macrophage migration inhibitory factor (MMIF) as putative cellular binding partners of **17i**.

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Introduction

Thiazolidinone derivatives are extremely potent class of biologically active heterocycles that are present as various drug molecules and natural products.[1-6] Representative examples include Pioglitazone (**1**) and its analogs, which are hypoglycemic and are administered orally to people suffering from type II diabetes, Epalrestat (**2**) an aldose reductase inhibitor, used in treating diabetes neuropathy, Darbufelone (**3**), a dual inhibitor of COX-2/5-LOX, used as an anti-inflammatory agent and etc (Figure 1).[7-9]

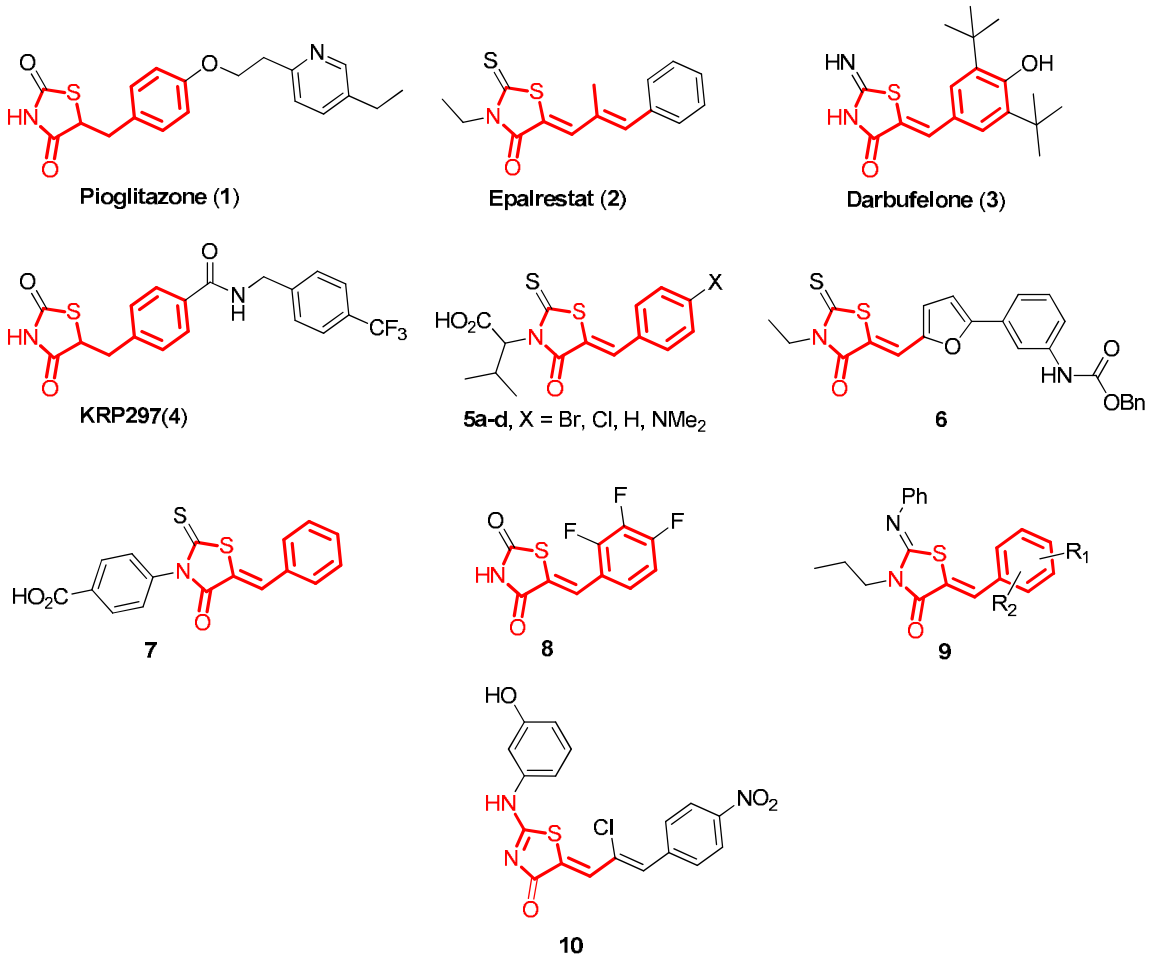

Figure 1. Representative examples of various thiazolidinones

In the recent times thiazolidinone molecules have exhibited interesting and potential anticancer properties. Compound **4**, displayed agonistic activities against peroxime proliferator-activated receptors (PPARS) that play vital role in cellular differentiation, development, metabolism and

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tumorigenesis in human beings, compounds **5-10** modulates anti-apoptotic proteins Bcl-X_L/BH_3, tumor necrosis factor TNFα, JSP-1, Pim-2 and Pim-1- protein kinases and COX-2 respectively. Recently Lesyk and co-workers investigated the potential of 5-alkenylthiazolidinone (**10**) as anticancer agents and discussed their structure activity relationship studies by screening them against 60 cancer cell lines of National Cancer Research Institute (NCI) (Figure 1).[10-16]

Among the thiazolidinone derivatives 5-arylidene-2-aminothiazolidinones are one of the most promising molecules in anticancer drug discovery process. In general they have exhibited significant antitumor activity (mean log GI_{50} ~5) against a bevy of cancer cells such as breast cancer, lung cancer and leukemia.[17]

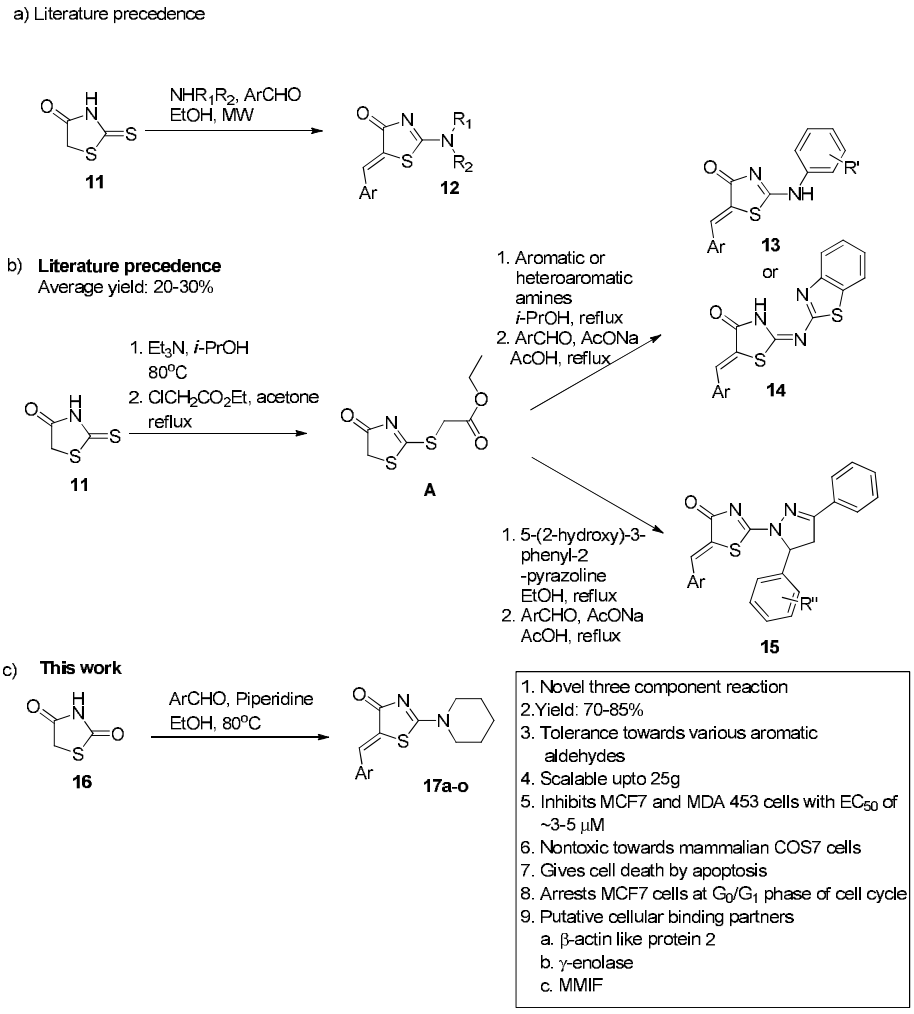

Figure 2. Synthesis of novel 5-arylidenethiazolidinones

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In conjunction to our group's systematic investigation towards new antiproliferative agents, here in we report a facile three component reaction to access new derivatives of 5-arylidene-2-aminothiazolidinones from thiazolidine-2, 4-dione, piperidine and appropriate aldehydes (Figure 2c). A similar one pot reaction was reported by Lesyk and co-workers with rhodanine (figure 2a).[18] Multistep synthesis of this class of compounds had been published earlier but in general the process' were not efficient as the overall yields were low (Figure 2b).[19-20] In comparison our strategy is simple, tolerate diverse functionalities and could be scaled up to 25g, using simple chromatographic separation technique or recrystallization for purification of the final product. Additionally, these compounds were found to be effective antiproliferative agents in human breast cancer (MCF7 and MDA453) cells. Detailed phenotypic profiling exhibited that these compounds give cell death by apoptosis and arrests the proliferation of MCF7 cells at G_0/G_1 phase of cell cycle. A novel chemical proteomic method based on affinity chromatography identified β-actin-like protein 2, γ-enolase and macrophage migration inhibitory factor (MMIF) as potential cellular binding partners of the most active compound **17i** (Figure 2c).

Results and discussions

To access the desired 5-arylidene-2-aminothiazolidinone we began the optimization of the three component reaction involving a one pot amine substitution and Knoevenagel condensation on the thiazoldine-dione**16**. For this reaction to work it is imperative that the amine that is to be installed on the thiazolidine-dione should also facilitate the Knoevenagel condensation. Initially we screened several amines that can act as a bifunctional reagent (Table 1, entry 1-7) on the model reaction between **16** (1 equiv.) and tolualdehyde (1 equiv.).And it turned out that piperidine was the only one to generate**17a** (44%, entry 3). On scanning the reaction in a bevy of solvents it was observed that ethanol was the best in affording **17a** (Table 1, entry 3 and 7-11), finally 3 equivalents of piperidine improved the yields from 44→80% (Table 1,entry 13) with no further improvement with the increase in equivalents (Table 1, entry 14).Conducting the reaction under milder reaction conditions such as at room temperature or at 40°C proved to detrimental to the yield (table 1, entry 15-16). Finally the best condition for this transformation was reacting **16** (1 equiv.), and tolualdehyde (1 equiv.) in presence of piperidine (3 equiv.) in ethanol as solvent under reflux for 12h.

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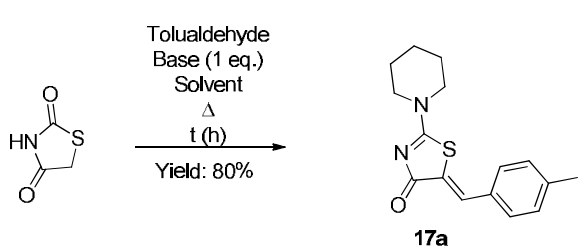
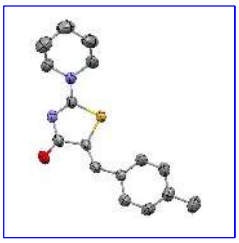
Table 1. Investigation of reaction conditions[c]





Entry	Base (eq.)	Solvent	Temperature (°C)	Yield(%)[a]
1	Morpholine (1)	Ethanol	Reflux	-
2	Cyclohexylamine (1)	"	"	-
3	Piperidine (1)	"	"	44
4	Pyrrolidine (1)	"	"	-
5	Diisopropyl amine (1)	"	"	-
6	Benzimidazole (1)	"	"	-
7	Piperidine (1)	Methanol	"	34
8	"	Isopropanol	"	40
9	"	Toluene	"	NR[b]
10	"	Dichloromethane	"	21
11	"	Acetonitrile	"	38
12	Piperidine (2)	Ethanol	"	65
13	**Piperidine (3)**	**"**	**"**	**80**
14	Piperidine (4)	"	"	76

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15	"	"	RT	NR[b]
16	"	"	40	53

[a] Isolated yield; [b] NR: No reaction; [c] Reactions were conducted with 40 mg of thiohydantoins

With the optimized condition for MCR in hand, next the generic nature of the protocol was investigated. Consequently various aldehydes were reacted under the reaction conditions and the results are depicted in figure 3. From the results it is conspicuous that the optimized methodology was compatible with aromatic and heteroaromatic aldehydes generating the desired products **17a-o** in excellent yields (Figure 3).

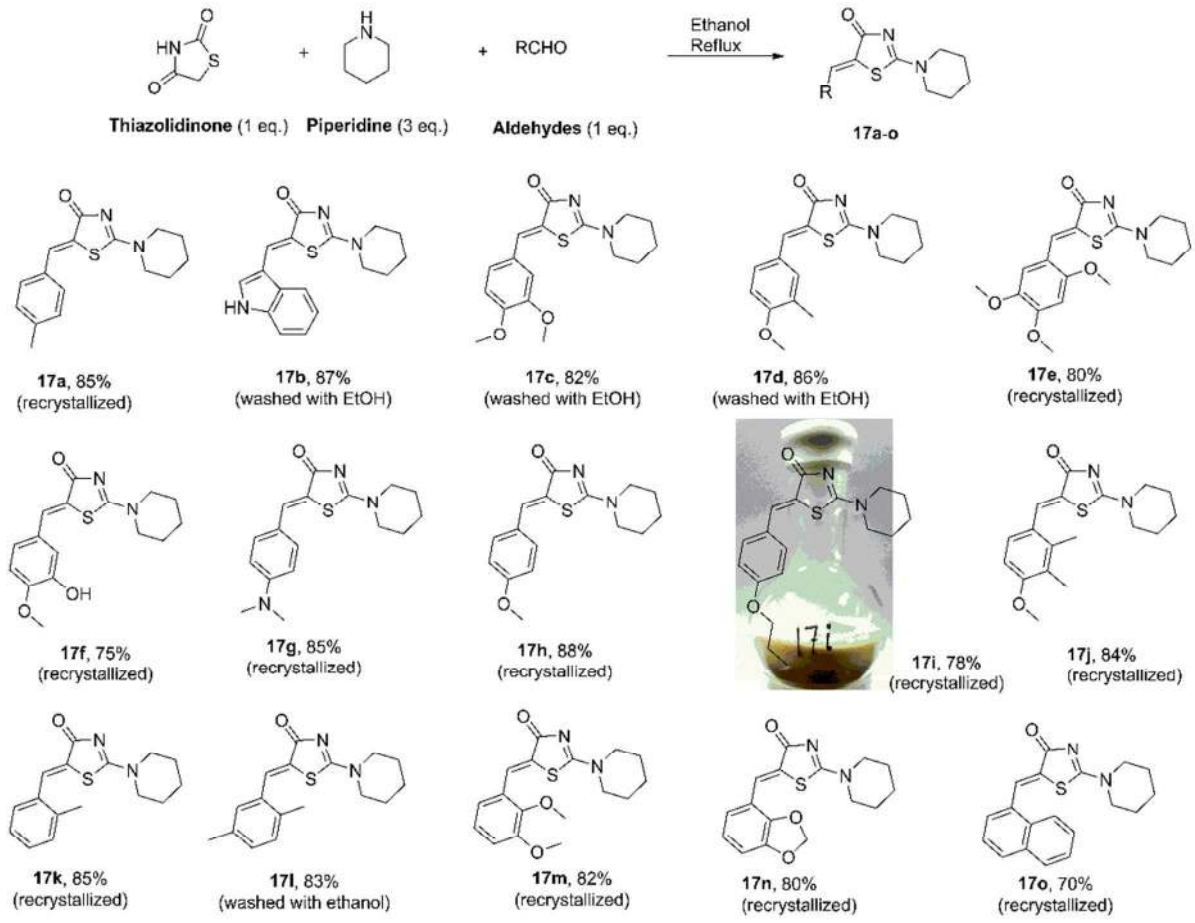

Figure 3. Generic nature of the multicomponent reaction towards the synthesis of 5-arylidenethiazolidinones **17a-o**.

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All the reactions are conducted on 200mg scaleas **17a**, **j**, **h**, **I** and **k** lead to the formation of 5-arylidenethiazolidinone in 60-78% yield. Multiple substitutions on the aromatic ring improved the yields to ~90% as exemplified by **17c-f**, **j**, **l-n**. The protocol worked efficiently for 3-indolecarbaldehydes as well as for napthaldehydes to afford **17b** and **17o** in 67 and 88% yields respectively. The compounds are characterized by ^{1}H and ^{13}C NMR spectroscopy, high resolution mass spectroscopy (HRMS) and single crystal X-ray crystallography (Figure 3).

To test the scalability of the protocol, 25g of **16** was reacted with *p*-*n*-propoxybenzaldehyde under the optimized condition to afford ~30g of the desired **17i** as yellow solid (65% yield) (Figure 3). The final product precipitated out from the reaction mixture which was purified by washing with ethanol.

A plausible reaction mechanism for the formation of 5-arylidenethiazolidinones is depicted in figure 4. The first step is assumed to be the piperidine promoted Knoevenagel condensation of **16** and *p*-tolualdehyde resulting in arylidenelthiazolidenedione **A**. The excess piperidine then initiates a nucleophilic attack on the thiocarbonyl carbon of **A** resulting to **B** which undergoes an elimination to generate the desired product **17a** (Figure 4).

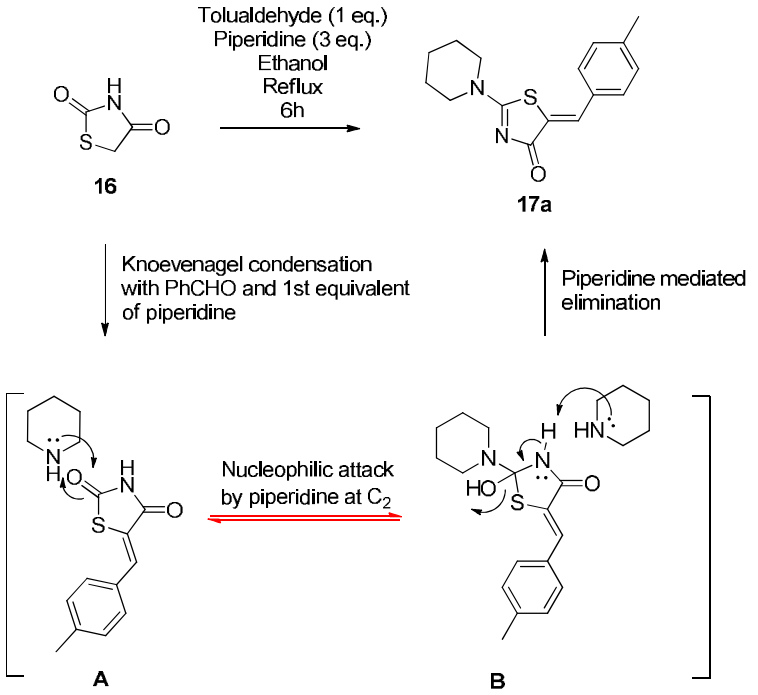

Figure 4. Plausible mechanism for the transformation of **16** to arylidenethiazolidinones**17**

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Biological evaluation. As mentioned earlier, thiazolidinones have a rich legacy of anticancer activities. Hence in order to assess their ability to inhibit cell proliferation of cancer cells our 5-arylidenethiazolidinone library was subjected to *in-vitro* cytotoxicity assay screening against human breast cancer cells such as MCF7 and MDA453. Preliminary structure activity relationship indicated that *para* substituents are tolerated followed by the combination of *meta*/ *para* substituents, whilst *ortho* substituents are not. Compound **17b**, which contained an indolyl moiety was moderately active and so was the naphthalyl analog **17o** (Table 2). Interestingly the *p*-anisolyl analog exhibited poorest activity among all the compounds. Finally,it was extremely gratifying to observe that several compounds of our library exhibited significant arrest of the cell proliferation of both MCF7 and MDA453 cells with ~ 50-67% inhibition (Table 2, **17c**, **e**, **g**, **i** and **m**). They further displayed the dose dependent inhibition with an EC_{50} of ~3.5 → 5μM in both MCF7 and MDA453 cells (Table 2). Etoposide was used as the positive standard. When screened against healthy human COS7 cell lines the average inhibition of the active compounds ranged from 8 → 17% which indicates that these compounds where not cytotoxic towards the COS7 cells (refer *supplementary information*).

Next **17i** was chosen as a representativemolecule for phenotypic profiling and molecular target identification.

Table 2. Antimitotic and growth inhibitory activity of MCR reaction products in MCF7 and MDA cells

Entry	Compound	Percentage inhibition[a]			EC_{50}(μM)[b]	
		MCF7	MDA	COS7	MCF7	MDA
1	**17a**	33.79±2.66	26.49±0.11	-	-	-
2	**17b**	37.45±0.07	45.43±0.02	-	-	-
3	**17c**	50.77±1.79	39.58±0.05	2.46±0.08	5.11	3.34
4	**17d**	43.5±2.05	52.31±0.26	8.96±0.08	4.64	3.15
5	**17e**	55.46±1.98	57.37±0.07	17.73±0.10	3.22	3.58

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6	**17f**	40.25±0.21	48.24±0.099	-	-	-
7	**17g**	56.78±1.75	49.86±0.06	9.88±0.05	3.64	4.06
8	**17h**	33.92±1.23	31.33±0.08	-	-	-
9	**17i**	66.4±1.22	60.03±0.09	8.32±0.03	4.42	3.5
10	**17j**	-	-	-	-	-
11	**17k**	44.35±0.37	30.93±0.05	-	-	-
12	**17l**	47.44±0.69	41.05±0.26	-	-	-
13	**17m**	54.67±1.65	27.15±0.18	-	-	-
14	**17n**	37.31±1.22	23.56±0.14	-	-	-
15	**17o**	44.46±0.997	45.65±0.08	-	-	-
16	**DMSO**	73.44	76.05±0.14	-	-	-
17	**Etoposide**	~100	-		2.36	-

[a] All data is the mean of one experiment conducted in triplicates. [b] EC_{50} of **17c-e**, **g** and **i** is the mean ± SD of three experiments

Epithelial cell migration is a regulated and harmonized mechanism in the cancer cells. It requires their chemotactic migration which is further guided by protrusive activity of the cellular membrane.[21] The conventional scratch wound motility assay exhibited the ability of **17i** to modulate migration of MCF7 cells in a denuded area.[22] For performing the assay, MCF7 cells were grown and after 24 h of seeding they were treated with 25 and 50 μM concentrations of **17i**. The cells were scratched by dragging a 100 μL pipette micro tip through the center of the plate. The cultures were cleaned and further treated with medium containing 5% FBS to facilitate cell migration. The cells were then incubated at 37°C for 24 h. They were photographed at 0, 12 and 24 h and the migrated distance was measured. The cell migration rate was obtained by counting five fields per image and the results presented as the average of two independent experiments performed over multiple days (Figure 5a). Analysis of the images showed that the **17i** transfected cells (Figure 5a) migrated much slower than the untransfected control cells (Figure 5a) thereby

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indicating inhibition of proliferation of MCF7 cells by **17i**. This information further corroborated the inhibitory potential of **17i** towards the proliferation of MCF7 cells. The cell migration in the above experiment is depicted graphically in Figure 5b.

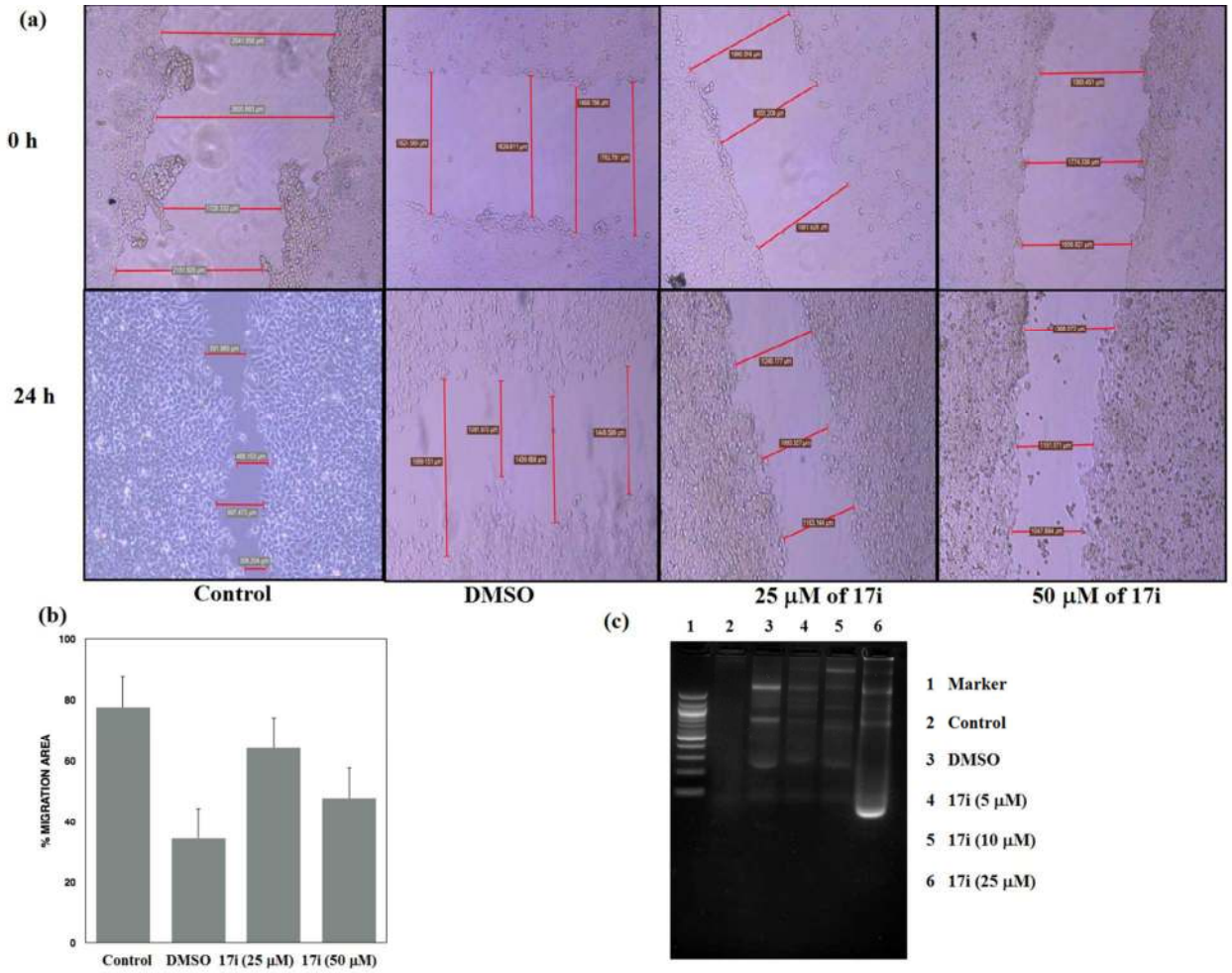

Figure 5. Cell motility studies. (a) Control without **17i**; DMSO; In presence of 25 µM of **17i**; In presence of 50 µM of **17i**; (b) The bar graph shows the percentage wound healing activity of the MCF-7 cells after treatment with **17i** at 25µM & 50µM as compared to the negative control (solvent alone) and positive control (DMSO). (c) DNA ladder assay depicting nucleosomal fragments of nuclear DNA. Lane 1: With marker; Lane 2: Control; Lane 3: with DMSO; Lane 4: **17i** (5 µM); Lane 5: **17i** (10 µM) and Lane 6: **17i** (25 µM).

The remarkable change of structural organization of nucleus of a cell is the apoptotic feature that discriminate it from the other types of cell death. During apoptosis fragmentation of nuclear

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proteins and DNA molecule result in chromatin condensation and formation of apoptotic bodies.[23] In our next experiment, nearly 50,000 MCF-7 cells were transfected with **17i** at varied concentrations such as5, 10 and 25 μM for 24 h and then DNA was isolated and analyzed by agarose gel electrophoresis. A typical ladder fragmentation pattern indicating nucleosomal fragments of nuclear DNA (an indication of apoptotic cell) was obtained in cells incubated with compound **17i**, with the most prominent formation at 25 μM.[24] No DNA inter nucleosomal fragmentation was observed in cells treated with 0.2% DMSO (vehicle control) (Figure 5c**)**. Hence this demonstrated that **17i** promotes apoptosis in MCF7 cells.

Next to determine whether apoptosis was the mechanism of cell death, we combined the assay of annexin V binding to recognize apoptotic cells with the stoichiometric staining of cellular DNA using propidium iodide (PI) to define the cell cycle position.[25] The experiment exhibited that treatment of MCF7 with **17i** induced apoptosis. As observed in Figure 6a below, the untreated cells showed no sign of apoptosis. However the cells treated with **17i** (at its EC_{50} concentration) (Figure 6a) shrank and their membranes lost integrity. First the Annexin V stain (green, 6h) became visible (indicating early apoptosis), next the cells became permeable and the PI gained access to the DNA as observed by the red coloration (12→24h) thereby indicating apoptosis.

Finally to examine whether the observed inhibition of cell growth involved changes in the cell cycle, we examined the cell cycle phase distribution by flow cytometry (Figure 6b and c).[26] The MCF-7 cells which were treated with **17i** were found to dose-dependently accumulate in the sub G_0/G_1 and G_0/G_1 phase, whereas the amount of cells in the G_2/M and S phase was significantly reduced (Figure 6b and c). Sub G_0/G_1 was represents the cell death whereas increased dose dependent G_0/G_1 population represents arrest in that phase (Figure 6b and c). In control cells, the proportion of sub G_0/G_1 cells was 2.6%, whereas on treatment with 3 and 6 μM**17i** treatment, the sub 24% and 43% respectively. In those doses, the G_0/G_1 population increased to about 72% and 85%, respectively, whereas in the control 60% of cells were in the G_0/G_1 phase (Figure 6b and c). These results suggest that **17i** arrest the progression of MCF-7 cells at G_0/G_1 phase of the cell cycle.

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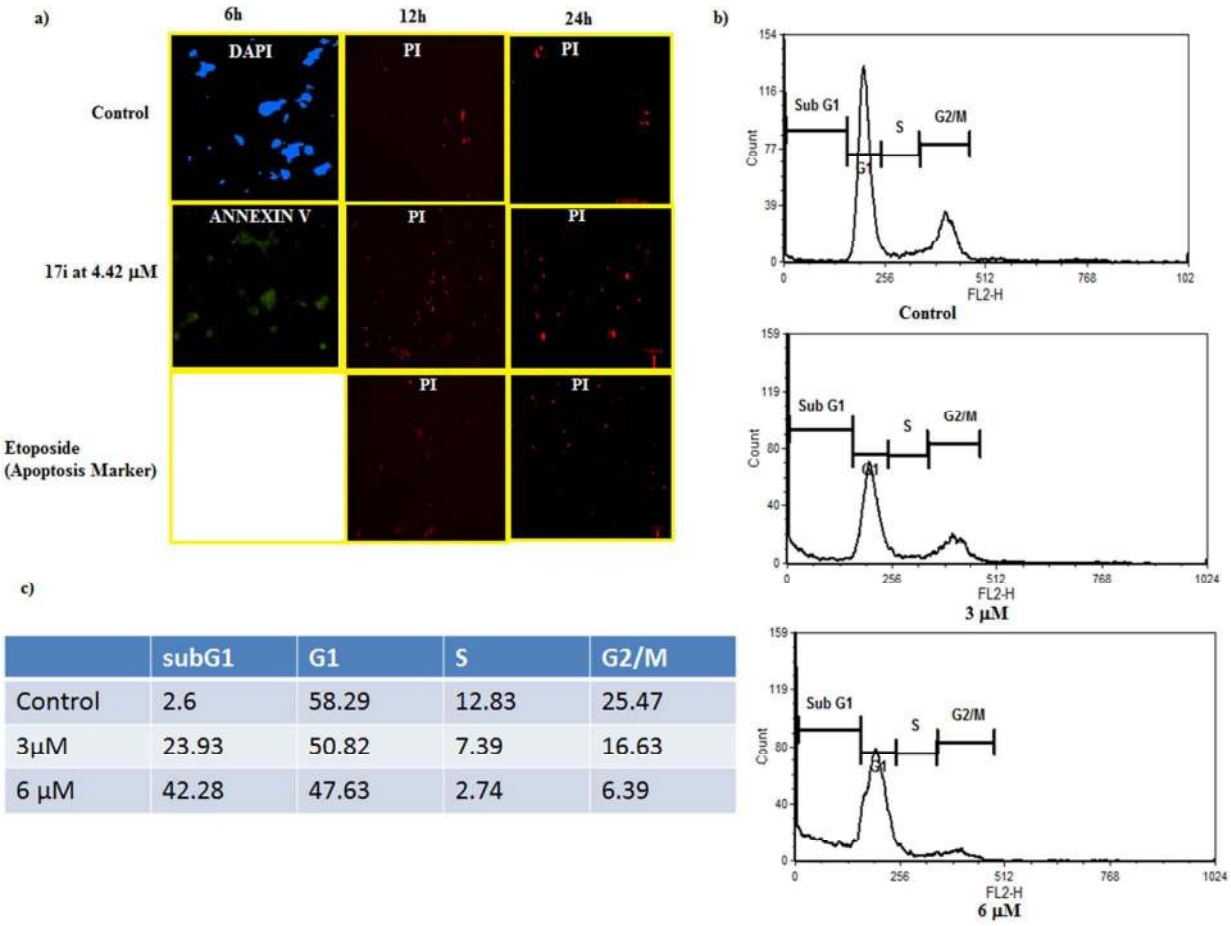

c)

	subG1	G1	S	G2/M
Control	2.6	58.29	12.83	25.47
3µM	23.93	50.82	7.39	16.63
6 µM	42.28	47.63	2.74	6.39

Figure 6. (a) Annexin V-PI assay; (b) and (c) Effect of **17i** on cell cycle distribution of MCF-7 cells (using total population)

Target identification. Deconvoluting molecular targets of active compounds from a phenotypic screening is an important step to understand the mechanism of action of the compound and also for harnessing the identified targets as devices for further understanding of the biological process. Hence to identify the molecular targets of **17i** a unique polymer based technology was used. In this validated method (please refer *SI*) an affinity matrix of **17i**, was prepared by immobilizing it on a polymeric surface with 40% methanol as a solvent. Immobilization is facilitated by multiple weak-intermolecular interactions between **17i** andthe polymer (prepared by doping chemicals that can provide possibilities of complementary weak-intermolecular interactions with **17i**, in nitrocellulose). In a typical procedure a 50 mM stock of **17i** in DMSO was diluted by 40% methanol to a concentration of 108 µM and incubated with the polymer for 16 h at room temperature followed by washing the resulting matrix with 1X TBS to remove any

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unbound molecule. To estimate the extent of immobilization **17i** was recovered from the matrix and quantified by mass spectrometry based method (scheme 4).Next, to capture the cellular binding partners of the molecule the affinity matrix was incubated with the MCF7 cell-lysate. To remove unbound proteins and to reduce non-specific interactions the matrix was washed with 1X TBS. The molecule bound proteins were then recovered by acetone precipitation and were dissolved in 1X sample buffer (Figure 7). In control experiments, the matrix (without **17i**) and the cell-lysate incubation were treated similarly. The gel-bands from the proteins were separated on a 4-12% gradient gel and commassie stained bands were digested by standard "*in-gel*" trypsin digestion method and were subjected to mass spectrometry analysis (refer *SI* for the details of the preparation) (Figure 7).

Agilent 6540 UHD Accurate Mass QTOF, set-up with Agilent 1260 infinity HPLC-CHIP cube system for sample infusion, was used for the mass-spectrometry analysis of the proteins. The tryptic peptides were loaded and eluted from HPLC-CHIP system. Mass data was collected in positive ion mode. Peptides identified were searched against the human database for the BLAST search (Figure 7).[27] To establish the identity of the protein a highly stringent criterion of 1% false discovery rate and identification of at least 2 unique peptides was chosen and data were filtered accordingly for the analysis. Proteins identified in the control and **17i** group was pooled together (refer *SI*). A total of 371 and 114 proteins were identified in control and **17i** groups respectively. Comparative analysis was performed to identify the proteins that were specifically identified in the **17i** group but not in the control group.

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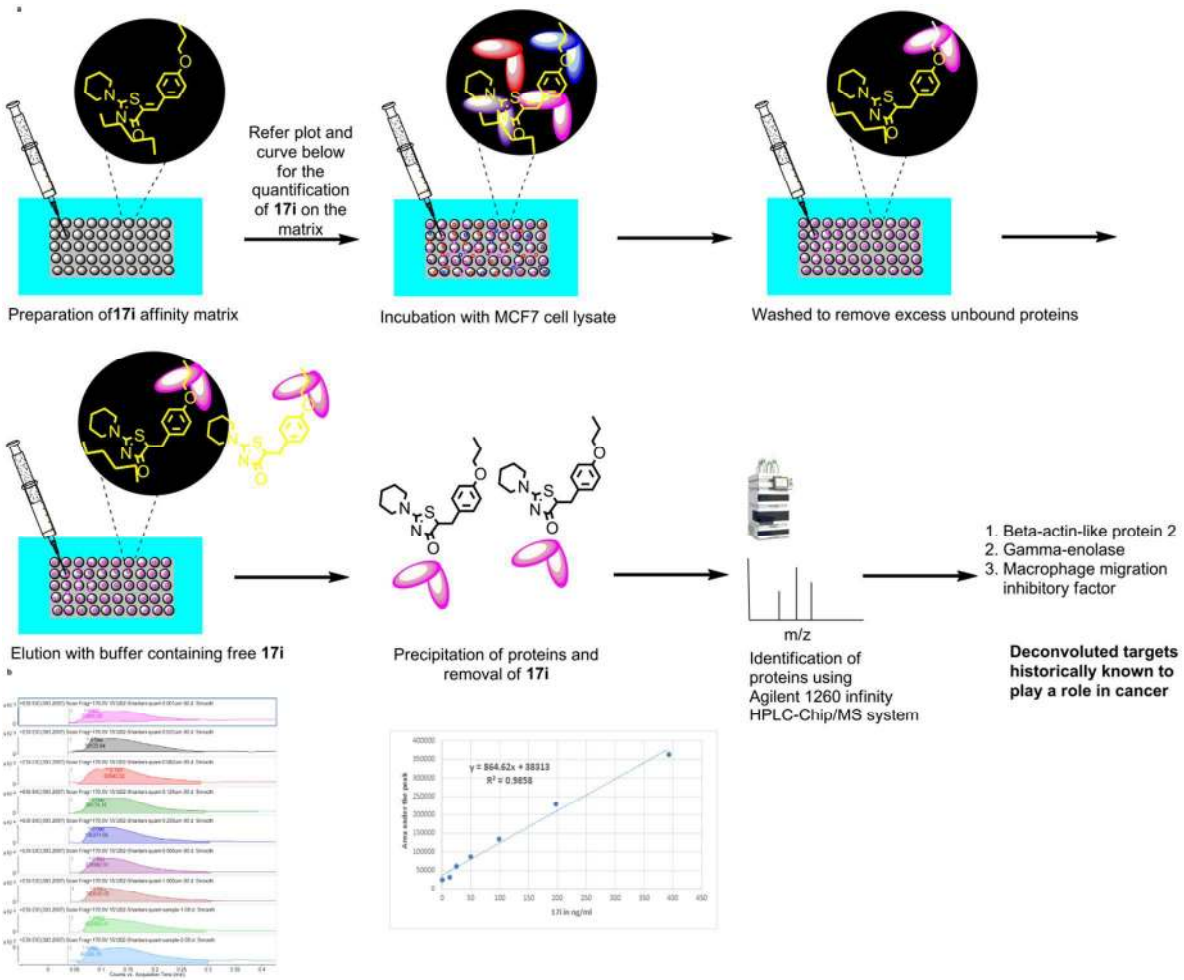

Figure 7. Work flow for target deconvolution of **17i** in MCF7

This analysis revealed a list of seven differential proteins (Table 3), the putative targets that were specifically present in the **17i** matrix. Proteins that are historically known to play a role in cancer are highlighted below in Table 3.

Table 3. Putative targets of **17i** in MCF7

S. No.	Uniprot ID	Protein Name	Protein Class	Number of Unique Peptide Detected	Summed Morpheus Score	Q-Value (%)
1	Q562R1	Beta-actin-like	Cytoskeletal	2	32.37	0

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		protein-2				
2	Q6ZN40	Tropomyosin 1 (Alpha), isoform	Cytoskeletal	2	22.27	0
3	P09104	Gamma-enolase	Enzyme	3	49.32	0
4	M0R0R2	40S ribosomal protein S5	Structural	2	31.24	0
5	P02768	Serum Albumin	Transport	2	27.18	0
6	P14174	Macrophage migration inhibitory factor	Inflammatory	2	24.32	0
7	B7Z6Z4	Myosin light polypeptide 6	Cytoskeletal	2	21.23	0

Conclusion

Here in, we have described atypical three component reaction strategy towards the synthesis of 5-arylidenethiazolidinone compounds from thiazolidine-2, 4-dione, piperidine and appropriate aldehydes. The piperidine played the role of a base to facilitate the Knoevenagel condensation between the thiazolidinedione and the aldehydes and also as functionality in the thiazolidinone ring. The compounds are obtained in high yields and are tolerant towards a variety of aldehydes thereby providing the target molecules in single step (in comparison to low yielding multistep syntheses reported in the literature). The desired products precipitate out of the reaction mixture and are isolated by filtration and purified by washing and recrystallization. These compounds revealed antimitotic activities against human breast cancer cells (MCF7 and MDA), that resulted in cancer cell death by apoptosis. Cell cycle analysis of the most active compound **17i** exhibited that the compound arrest the progression of MCF-7 cells at G_0/G_1 phase.They are nontoxic towards mammalian COS7 cells. A novel chemical proteomics approach based on affinity chromatography identified β-actin-like protein 2, γ-enolase and macrophage migration inhibitory

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factor (MMIF) as putative cellular binding partners of **17i**, thereby providing the molecular basis for the potent anti-proliferative activity of this class of molecules.

Presently in our lab, we are conducting structure activity relationship studies between our newly discovered 5-arylidene-2-amino thiazoldinones and the enzyme targets to improve the potency and identify a candidate suitable for animal study.

Experimental

Chemical synthesis. Compounds were synthesized according to procedures specified in Supplementary Methods. X-Ray crystallographic data and images are reported in Supplementary Fig. 6. For ^{1}H and ^{13}C NMR spectra of compounds reported see Supplementary File.

Cell culture conditions. MCF7 cells were maintained as monolayer in 25 cm^2 culture flasks (T-25) at 37°C in DMEM medium (GIBCO, St. Louis, USA) supplemented with high glucose,L-glutamine,pyridoxine hydrochloride,110 mg/L sodium pyruvate, 3.7 g/L NaHCO3 and antibiotics (Penicillin-10,000 units/mL, Streptomycin -10,000 µg/mL, Gibco USA). Growth medium (pH 7.4) was prepared by adding 5% heat inactivated FBS (Fetal Bovine Serum, Gibco, USA) and stored at 4°C. The medium was changed 10–18 h prior to experiment, and cells were confluent at the time of experimentation. Cells were dislodged from flasks by gentle trypsinization containing 0.25 % Trypsin. MCF7 cells were regularly sub-cultured thrice a week in a seeding density of 40,000–50,000 cells/cm^2area.

Cell Viability Assay (MTT Assay). The MTT colorimetric viability assay was performed to evaluate the anticancer effect of compounds. 30,000 cells/100 µl of media were seeded per well and allowed to stretch and adhere overnight at 37°C. Following day media was removed and 100 µl fresh media was added to the grown cells. Adhered cells were then treated with the compounds **17a-o** dissolved in DMSO in triplicates at a standard concentration of 50 µM for 48 hours. Cytotoxic effect was evaluated using ability of live cells to cleave MTT ((3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide)) (Sigma-Aldrich, St Louis, MO, USA), into formazan crystals. Post 48 hours of treatment, 10 µl of MTT (5 mg/ml in PBS) was added to the cells and incubated for 4 hours in dark at 37°C. The violet colored crystals formed were then dissolved in 100 µl DMSO solvent (Dimethyl Sulfoxide, Sigma-Aldrich, St. Louis, MO, USA). The colorimetric assay was read measured by a MultiModePlate Reader (Bio-Rad) at 595nm. Percentage of Inhibition of drug was calculated by following formula:

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(Average of control $O.D_{595}$-Average of Treated $O.D_{595}$) / control $O.D_{595}\times100$.

Cell Migration assays.MCF7 cells were grown to confluence in six-well tissue culture plastic dishes to a density of ~0.2 million cells/well. After 24 h of seeding, cells were treated with chemical compounds. The medium was removed and the cells were scratched by 200 µL pipette micro tip through the center of the plate. The cultures were washed to remove debris and re-cultured with DMEM containing 10% FBS for cell migration. The cells were imaged at 24 h and the migrated distance was measured. Migration rate was calculated by counting five to seven fields per image and the results presented as the average of three independent experiments performed over multiple days.

DNA fragmentation assay. The DMSO (dimethyl sulphoxide)–SDS (sodium dodecyl sulphate)–TE (Tris-EDTA) method: 2 million MCF7cells were taken as starting material for DNA isolation. Cells were dislodged with Trypsin-EDTA and washed with 1 X PBS.DMSO was added directly to the cell pellet (100µl) and mixed well by pipetting. TE buffer (pH 7.4) with 2% SDS was added in to the mixture with same volume, followed by pipetting. The solution was centrifuged at 3000 rpm at 4°C and 30 µL of the supernatant was loaded on agarose gel to check the effect of drugs on DNA molecule.

Annexin V/ propidium iodide staining. The assay is based on the ability of the protein annexin V to bind to phosphatidylserine (PS) exposed on the outer membrane leaflet in apoptotic cells (PS also appears on the necrotic cell surface). The annexin V assay was performed following the manufacturer's instructions (Annexin V-FITC kit,Alexa Fluor). Briefly, the cells were washed with PBS and suspended in pre-diluted binding buffer. The cell density was adjusted to 2 million and 195 µl of cell suspension were mixed with 5 µl Annexin V-FITC and incubated for 10 min in the dark at room temperature. The cells were washed with binding buffer, re-suspended in 195 µl binding buffer, counterstained with 10 µl of the 20 µg/ml propidium iodide stock (final concentration 1 µg/ml) and examined under the fluorescence microscope.

Cell Cycle Phase Distribution Analysis. The effect of different ligands on the progression of MCF-7 cells throughthe cell cycle was monitored by flow cytometry using a BectonDickinson FACS Calibur instrument. The cell cycle distributionpatterns were determined after processing of treated cells withRNase A (1mg/ml) followed by staining with PI.

Affinity Chromatography and Enrichment of Cellular Binding Partner.The cellular binding partners, the targets, of the molecule were enriched using Shantani's Proprietary UPT method (Pro. Patent Application # 201621000681).[28] In this validated method (Supplementary Information) an affinity matrix

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of the molecule, without its derivatization, is prepared by immobilizing the molecule on a polymeric surface using multiple weak-interactions of the molecule with the polymer. Immobilization of the molecule over the polymer matrix is confirmed either by quantifying the amount of the molecule in extensive matrix washes that are carried out after molecule immobilization or by quantifying the amount of the molecule retained over the matrix by recovering the molecule from the matrix after extensive wash. Thus prepared 'molecule specific affinity matrix' is then incubated with the cell-lysate to capture the cellular binding partners of the molecule. For enriching targets of **17i**,a 50 mM stock of **17i** was prepared in DMSO. The stock solution was diluted in either 40% methanol or 100% chloroform to a concentration of 108 μM and incubated with the matrix for 16 h at room temperature. After incubation, the matrix was washed thrice with 1X TBS to remove any unbound molecule. To estimate the extent of immobilization the molecule was recovered from the matrix and quantifies by mass spectrometry based method. Cell lysates were prepared in RIPA buffer and protein concentration was determined by Bradford method. Cell lysate was diluted with 1X TBS to a concentration of 1 mg/ml. The molecule immobilized matrix was incubated with 2 ml of cell lysate for 2 min with mild shaking. To remove unbound proteins and to reduce non-specific interactions the matrix was washed with 1X TBS. The molecule bound proteins were then recovered by acetone precipitation and were dissolved in 1X sample buffer. In control experiments, T-4Pro was not immobilized on the matrix and the matrix and the cell-lysate incubation were treated similarly.

Sample Preparation for Mass Spectrometry Analysis. Proteins obtained from affinity chromatography experiments were separated on a 4-12% gradient gel and commassie stained bands were digested by standard *"in-gel"* trypsin digestion methods.[29]Clearlyidentified gel-bands were cut in typical size of 1 mm band from the gel and placed in 1.5 ml tubes. Gel slices were washed once with distil water and then twice, with 100 μL of 100 mM Ammonium Bicarbonate / 50% Acetonitrile solution by placing the bands in each respective solution at room-temperature for 30 minutes. After discarding the last wash gel-slices were incubated with 50 μL of 10 mM DTT at 56˚C for 10 minutes in a dry bath. Tubes were then cooled and then gel-slices were incubated with 50 μL of 100 mM IAA at 37˚C for 30 minutes. IAA solution was removed and gel slices were rinsed by incubating it with 100 mM Ammonium Bicarbonate / 50% Acetonitrile solution at 37˚C for 15 minutes. Rinse solution was removed and gel-slices were dehydrated by incubating them with 100 μl of 100% Acetonitrile for 10 minutes. Acetonitrile was removed and gel-slices were dried using a Speed Vac for 45-60 minutes at room temperature. Gel-slices were then incubated with 20-30 μl of 20 μg/ml trypsin solution for an hour at room temperature. 50 μl of 50 mM Ammonium Bicarbonate was added to the gel-slices and completely covered tubes were placed at 37˚C for 12 hours for maximum trypsin digestion of the protein. Gel-slices were then incubated with 150 μl of

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HPLC water for 10 minutes. Solution was frequently mixed using vortex mixer. Supernatant was removed and saved after appropriate labelling. Gel-slices were then incubated with 100 µl of 50% Acetonitrile /5% TFA at 37˚C for 60 minutes to extract the peptides from gel-slices. Supernatant was collected and pooled with earlier collected supernatant. Supernatant was then concentrated at room temperature until moderate dryness. Dried peptides were dissolved in 5 µl of 0.1%TFA/HPLC Water solution and set-aside for mass-spectrometry analysis.

Mass-Spectrometry Analysis of Protein. Agilent 1260 infinity HPLC-Chip/MS system is a microfluidic chip-based technology that incorporates peptide enrichment and separation and provides high-sensitive nanospray. The tryptic Peptides were loaded and eluted from HPLC-Chip system were eluted using a linear gradient of Acetonitrile (0 to 40% over the period of 60 minutes: Solvent A: 0.1% Formic Acid, Solvent B: 90% Acetonitrile / 10% (0.1% Formic Acid)) and directly infused into mass-spectrometer for detection using a flow rate of 0.3 µl/min. MS were scanned in the range 275-1700 m/z with scan rate 8 spectra/sec and MS/MS Scan rate was kept at 3 spectra/sec. Mass data was collected in positive ion mode at fragmentor voltage of 170V and Skimmer Voltage of 65V.

Spectral Analysis, Protein Database Searches, Protein Identification and Target Deconvolution.Agilent Mass Hunter software was used for data acquisition and analysis of Total ion chromatograms. Protein searches were carried out using Morpheus software tools using the Human proteome database. Auto-validation protocol having < 1% False Detection Rate was used in confirming the identity of protein. Further, protein identified with at least 2 unique peptides was considered as 'identified protein' and used in downstream analysis. Proteins (a) identified in duplicate experiments where **17i** molecule was immobilized but not identified in control experiments (where **17i** was not immobilized on matrix) and (b) identified in significantly higher amount in duplicate experiments where **17i** was immobilized compared to control experiments were considered as putative targets of **17i.**

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