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Structure–activity relationship study of 4EGI-1, small molecule eIF4E/eIF4G protein–protein interaction inhibitors

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ABSTRACT

Protein–protein interactions are critical for regulating the activity of translation initiation factors and multitude of other cellular process, and form the largest block of untapped albeit most challenging targets for drug development. **4EGI-1**, (*E*/*Z*)-2-(2-(4-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, is a hit compound discovered in a screening campaign of small molecule libraries as an inhibitor of translation initiation factors eIF4E and eIF4G protein–protein interaction; it inhibits translation initiation *in vitro* and *in vivo*. A series of **4EGI-1**-derived thiazol-2-yl hydrazones have been designed and synthesized in order to delineate the structural latitude and improve its binding affinity to eIF4E, and increase its potency in inhibiting the eIF4E/eIF4G interaction. Probing a wide range of substituents on both phenyl rings comprising the 3-phenylpropionic acid and 4-phenylthiazolidine moieties in the context of both *E*- and *Z*-isomers of **4EGI-1** led to analogs with enhanced binding affinity and translation initiation inhibitory activities.

1. Introduction

Alleviation of physiological constraints on cap-dependent translation plays critical roles in many pathological processes such as Wolcott–Rallison syndrome, fragile X syndrome [1], neurodegenerative disorders such as Alzheimer's disease and proliferative disorders such as cancer [2,3]. Interaction of eIF4E with mRNA Cap structure is a critical step in cap-dependent translational initiation. Eukaryotic translation initiation factors (eIFs)-mediate the assembly of the 43S pre-initiation complex at the 5′ Cap-mRNA enabling the 5′-to-3′ scanning of the 5′ untranslated region (UTR). Upon reaching the AUG initiation codon the 43S ribosomal subunit is joined by the 60S subunit and forms the 80S initiation complex. The eIF4E, which binds to the Cap-mRNA, is the least abundant initiation factor [4,5]. It forms a heterotrimeric eIF4F complex with eIF4G, the scaffolding protein, and eIF4A, the RNA helicase [6,7]. Assembly of the eIF4F complex involves critical protein–protein interactions (PPIs) and is regulated by competition for binding to eIF4E between eIF4G and eIF4E binding proteins (4E-BPs). Phosphorylation of 4E-BPs through the phosphatidylinositol 3-kinase/AKT/mTOR pathway reduces their affinity for eIF4E, enables eIF4E/eIF4G interaction, and subsequently the assembly of the eIF4F complex.

Targeting PPIs that mediate many important biological processes, like the interaction between eIF4E and eIF4G that is critical for the cap-dependent translation, by small molecule inhibitors is an emerging and challenging area in drug design. Contrary to enzymes and receptors that are characterized by well-defined binding-sites, which can avidly interact with small molecule ligands, and are therefore recognized as traditional targets for drug development, PPI interfaces are large, ill-defined and in general featureless. Therefore, development of small molecules that can effectively disrupt a PPI is still an arduous and low-yield process.

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Nevertheless, successful development of potent and specific inhibitor of drugable PPIs promises to be a highly rewarding therapeutic endeavor.

Our laboratories have previously reported a high throughput fluorescence polarization (FP) assay for discovery and development of agents that disrupt eIF4E/eIF4G interactions [8]. Screening chemical libraries of small molecules have led to the discovery of a prototypic inhibitor of eIF4E/eIF4G interaction; **4EGI-1** (Fig. 1). **4EGI-1** inhibits expression of regulatory proteins such as cyclin E, cyclin D1, c-myc and Bcl-2 without any apparent effect on the expression of housekeeping proteins [9]. Recent studies utilizing **4EGI-1** as a molecular probe have made a significant contribution to our understanding of the role of eIF4F in memory formation and consolidation [10], autism spectrum disorders [11] and viral infection [12].

Despite its uses as a molecular probe, **4EGI-1** is in need of optimization to improve its binding affinity, solubility, and configurational lability. To this end, we conducted extensive structure–activity relationship studies on the **4EGI-1** and reported preliminary results in several meetings [13–17]. In here, we report our exhaustive efforts to delineate the putative pharmacophore and gain insight on the structural latitude to improve potency and physicochemical properties.

2. Results and discussion

2.1. Molecular design

Our initial studies of **4EGI-1** (Fig. 1) identified the carboxyl group, hydrazono function, thiazolidine ring, and the benzyl and phenyl moieties as essential components in the minimal pharmacophore of this (E/Z)-2-(2-(4-(3,4-dichlorophenyl)thiazol-2-yl) hydrazono)-3-(2-nitrophenyl)propanoic acid [13–17]. Reduction of the imino or replacing the hydrazono with amino have abrogated the activity, whereas, removal of the benzyl CH_2 in **4EGI-1** retained its activity. Attempts to replace the thiazolidine ring with triazole or oxazole have resulted in diminished potency, whereas hydrophobic substituents on position 5 of the thiazolidine ring were well tolerated [13–17]. Herein, we study the relationship between the nature of the substituents on both phenyl rings (**A** and **C** in Fig. 1) and their contribution to the biological activities such as cell-free and cell-based translation initiation specific assays. We sample a wide range of substituents that include substituents with various chemical properties such as electron-withdrawing, electron-donating, hydrogen bonds donors and acceptors, potential charge bearing basic and acidic moieties, as well as non-charged hydrophobic and hydrophilic moieties.

2.2. Synthesis

Derivatives of **4EGI-1** were synthesized following two different routes (Scheme 1): A) Condensation of either α-keto acid, **5**, or the 2-hydroxyacrylic acid, **4**, with preformed 2-hydrazinyl-4-phenylthiazoles, **9**, which was obtained via a Hantzsch type cyclodehydration of α-bromoacetophenones, **7**, with thiosemicarbazide, to form the anticipated **4EGI-1** derivatives, **10**–**12** (Scheme 1, route A); and B) a Hantzsch type cyclodehydration of the appropriate α-bromoacetophenones, **7**, with a substituted (2-carbamothioylhydrazono)-3-phenylpropanoic acid, **8**, forming the anticipated derivative of **4EGI-1** (Scheme 1, route B). The structural identity and integrity of all final **4EGI-1** derivatives was confirmed by HR-MS, ^1H , and ^{13}C NMR. The purity of the compounds exceeded 95% as determined by RP-HPLC.

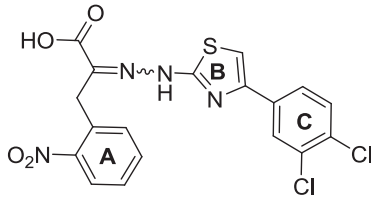


Fig. 1. Structure of **4EGI-1**.

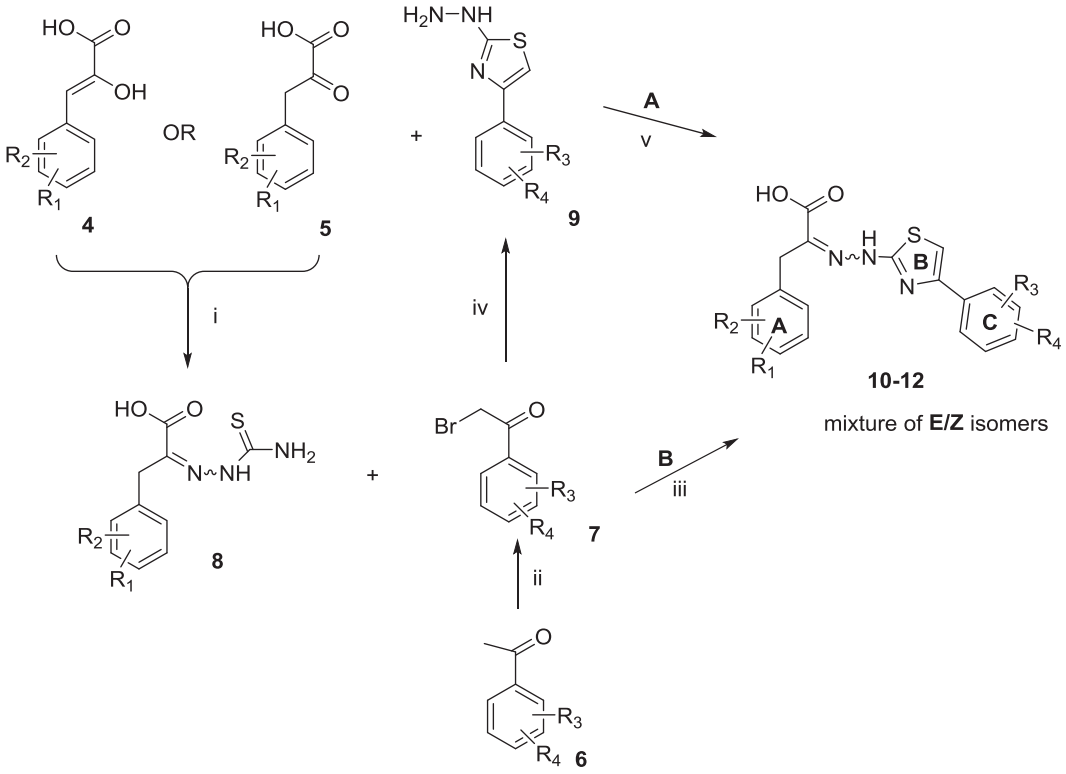
Ring **C** modified **4EGI-1** analogues were generally synthesized following route-A using either commercially available or in-house synthesized α-bromoacetophenones as described in the Experimental Section following known procedures [18]. Ring **A** modified **4EGI-1** analogues were generally synthesized following route-B. The 2-hydroxyacrylic acids, **4**, were synthesized from the appropriately substituted benzaldehydes, **1**, following the classical transformations as shown in Scheme 2. The di-substituted 2-hydroxyacrylic acids, **4h**–**m**, which were not commercially available, were synthesized from the appropriately *p*-substituted benzaldehydes, **1**, by nitration followed by the classical transformations (Scheme 2). The α-keto acids, **5**, were purchased from commercial sources or synthesized following previously described procedures [19].

2.3. E- and Z-isomers

RP-HPLC, LC–MS and high-resolution ^1H and ^{13}C NMR spectroscopy revealed that the non-regioselective syntheses of **4EGI-1** analogs described above result in mixtures of *E*- and *Z*-isomers around the imino, C=N-bond (Fig. 2). Almost exclusively, the *Z*-isomer is the predominant one in the reaction mixtures and based on RP-HPLC it is always the more hydrophobic one. The two isomers can be separated by RP-flash chromatography (Methanol/Water/0.1% Formic acid) and stored at 4 °C as stable solids for several months. Pure *E*-isomers undergoes *E*-to-*Z* isomerization at different rates and generate different equilibrium mixtures when dissolved in polar solvents [20]. These variations might be due to the different nature of substituents on rings **A** and **C**. This isomerization is enabled by the azo-hydrazone tautomerism as illustrated in Scheme 3. The greater thermodynamic stability and larger hydrophobicity of the *Z*- versus the *E*-isomer can be attributed to the exclusive capacity of the *Z*-isomer and its precursor *E*-azo tautomer to form intramolecular 6-membered hydrogen bonded rings (Scheme 3 and Fig. 2). Unlike *E*-isomer, the *Z*-isomer undergoes intramolecular hydrogen bonding that is enabled by the proximity of the hydrazono NH to the CO_2H group, which in turn, competes with the formation of intermolecular hydrogen bonds between two molecules of the [*Z*]-**4EGI-1** analogue and/or a molecule of the [*Z*]-**4EGI-1** analogue and solvent molecules. This difference in hydrogen bond capacity results in the distinct physical properties of the two isomers of **4EGI-1** and its analogues that includes polarity, solubility and melting points.

Except for compounds **12f** in which we could not separate the isomers by reversed phase column chromatography, and **12h**; in which the obtained precipitate was the pure *Z*-isomer, all the other **4EGI-1** analogues reported inhere were successfully separated by reversed phase column chromatography as pure *E*- and *Z*-isomers.

The *E*- and *Z*-isomers of a **4EGI-1** analogue differ in their ^1H NMR and ^{13}C NMR spectra (Figs. 2 and S1 and Table 1). The most distinctive ^1H NMR signals were the benzylic $C\underline{H}_2$ singlet ($H_{\boldsymbol{b}}$) attached to C=N, and the $C\underline{H}$ singlet ($H_{\boldsymbol{c}}$) in the thiazolidine ring, which were shifted up-field in the *Z*- relative to the *E*-isomer (see Fig. S1, Table 1 and Experimental Section). The equilibrium



[a] Reagents and reaction conditions: **i**) thiosemacarbazide, 1 equiv., 5% AcOH/ DDW , reflux, 2 hrs. **ii**) Br_2, AcOH, RT. **iii**) 1,4-dioxane, RT, 8 hrs. **iv**) thiosemicarbazide, 1 equiv., 1,4-dioxane, RT, 8 hrs. **v**) 5% AcOH/ethanol, reflux, 1 h.

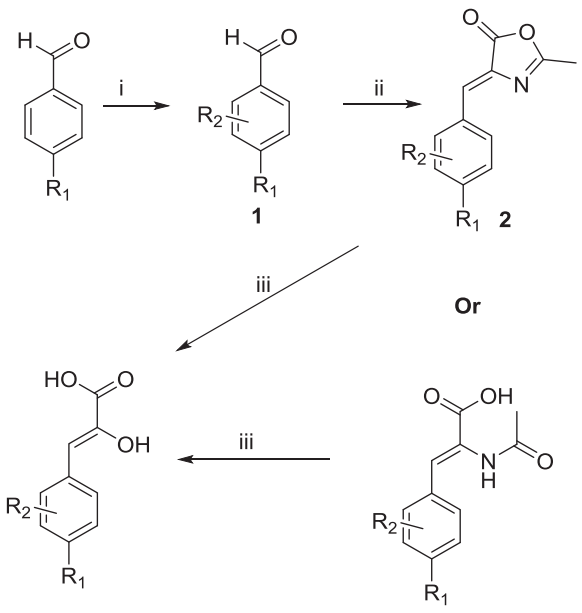
Scheme 1. Schematic synthetic pathways for the preparation of **4EGI-1**-derived thiazol-2-yl hydrazones[a].

between *E*-isomer and its diazo-tautomer cause delocalization of the thiazolidine ring π-electrons due to the extended conjugation systems, leading to a de-shielding effect and up-field chemical shifts for the protons involved. In addition, due to the spatial proximity of the aryl *o*-$C\underline{H}_a$ on phenyl ring **A** to the hydrazone doublet NH in the *E*-isomer the H_a is shifted downfield relative to the same hydrogen in the *Z*-isomer. The difference in the chemical shifts of these protons ranged from 0.1 to 0.5 ppm. In the ^{13}C NMR, the chemical shifts of benzylic and thiazolidine CH carbons were also affected. The most notable was the benzylic $\underline{C}H_2$ (C_b) that was shifted 2–9 ppm downfield in the *Z*-isomer relative to *E*-isomer. These changes reflect the sensitivity of the ^{13}C shielding constant to the spatial orientation of the imino nitrogen lone pair $=\ddot{N}$. The α-imino carbon in the *trans*-configuration is always shielded as compared with that in the *cis*-configuration [21]. These effects can be successfully used for configurational assignment of the isolated thiazol-2-yl hydrazones isomers. The high chemical shift of the hydrazones NH proton in the *Z*-isomers, which is greater than 12.5 ppm, indicates a strong de-shielding effect due to its involvement in an intramolecular NH···O=C hydrogen bond formation [22]. Confirmation of the configurational assignment of *E*- and *Z*-isomers, as well as the presence of NH···O=C intramolecular hydrogen bonding was obtained by the solid phase single crystal X-rays analyses (Data not shown).

2.4. Structure–activity relationship

For SAR studies we employed our previously described fluorescence polarization (FP)-binding assay [8], which reports on the displacement of fluoresceinyl-labeled eIF4G-derived peptide that contains the consensus eIF4E-binding sequence by a small molecule ligand. Binding of the labeled peptide to recombinant eIF4E, which was expressed as GST fusion protein and included a deletion of the first 26 amino acids (GST-ΔN26-eIF4E), generates a strong polarized fluorescent signal. In the presence of a small molecule (i.e. **4EGI-1** analogues) that competes for binding to eIF4E the intensity of the polarized light is reduced. We present these results as a ratio between the IC_{50} of [*Z*]**-4EGI-1** defined as the concentration of [*Z*]**-4EGI-1** needed to displace 50% of the fluorescent-tagged peptide, and the IC_{50} of the new **4EGI-1** analogue, which is the concentration of the new analogue needed to displace 50% of the fluorescent-tagged peptide when measured in the same 384-well plate (Tables 2–4).

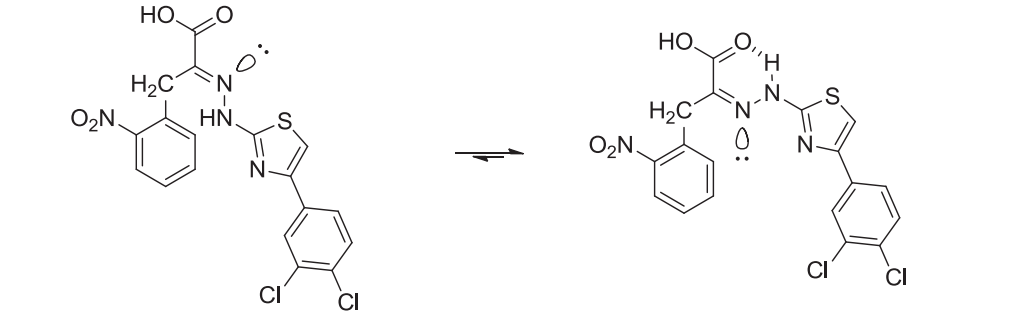
The affinity of both *E*- and *Z*-isomers of **4EGI-1** and its derivatives to the target protein eIF4E as measured by the FP assay is very similar (Tables 2–4), but when one isomer is more potent it is always the *Z*-isomer. Among the ring **A** mono-substituted **4EGI-1** analogues the nitro-derivatives were the most potent ones displaying very similar affinities in which *o*- ≥ *p*- > *m*-NO_2 (Table 2, cf. [*Z*]**-4EGI-1**, [*Z*]**-10b**, and [*Z*]**-10a**). Importantly, non-substituted or mono-substitution with of halides, carboxyl, and amino



1a: R_1= -NH-Ac, R_2= *m*-NO_2
1b: R_1= -CO_2H, R_2= *o*-NO_2
1c: R_1= -Cl, R_2= *m*-NO_2
1d: R_1= -F, R_2= *m*-NO_2
1e: R_1= -MeO, R_2= *m*-NO_2
2a: R_1= NH-Ac, R_2= -H
2b: R_1= F, R_2= -H
2c: R_1= -CO_2H, R_2= *o*-NO_2
2d: R_1= -OH, R_2= *m*-NO_2
2e: R_1= -Cl, R_2= *m*-NO_2
2f: R_1= -F, R_2= *m*-NO_2
2g: R_1= -OMe, R_2= *m*-NO_2
2h: R_1= -CO_2H, R_2= -H
2i: R_1= -NH-Ac, R_2= *m*-NO_2
3a: R_1= -H, R_2= *m*-NO_2
3b: R_1= -H, R_2= *p*-NO_2
3c: R_1= -Cl, R_2= -H
3d: R_1= -CF_3, R_2= -H
4a: R_1= -H, R_2= *m*-NO_2
4b: R_1= -H, R_2= *p*-NO_2
4c: R_1= -CO_2H, R_2= -H
4d: R_1= -NH_2, R_2= -H
4e: R_1= -Cl, R_2= -H
4f: R_1= -F, R_2= -H
4g: R_1= -CF_3, R_2= -H
4h: R_1= -NH_2, R_2= *m*-NO_2
4i: R_1= -CO_2H, R_2= *o*-NO_2
4j: R_1= -OH, R_2= *m*-NO_2
4k: R_1= -Cl, R_2= *m*-NO_2
4l: R_1= -F, R_2= *m*-NO_2
4m: R_1= -OMe, R_2= *m*-NO_2

[a] Reagents and reaction conditions: **i**) 1.1 equiv. of HNO_3/H_2SO_4, 0°C, 30 min. or 1.1 equiv. of NO_2BF_4/ ACN, 0°C, 30 min. **ii**) 1.2 equiv. of acetic anhydride, 5 equiv. of N-acetyl glycine and 5 equiv. of sodium acetate, reflux for 4 hrs. **iii**) 32% $HCl_{(aq)}$, reflux, 1-2 hrs.

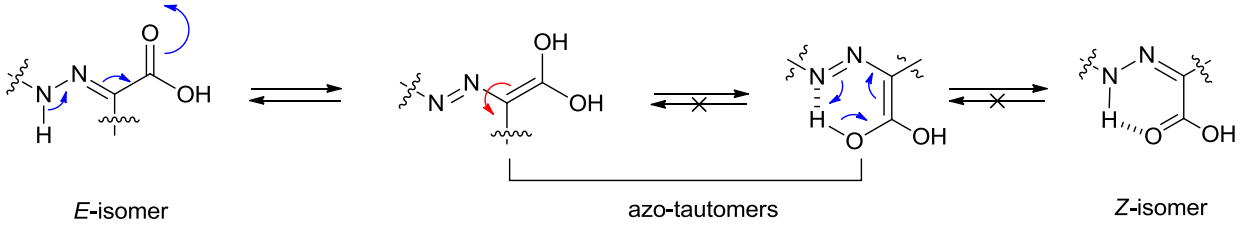
Scheme 2. Schematic synthetic pathways for preparation of phenyl substituted 2-hydroxyacrylic acids **4**[a].



(*E*)-2-(2-(5-(3,4-dichlorophenyl)thiazol-2-yl) hydrazono)-3-(2-nitrophenyl)propanoic acid

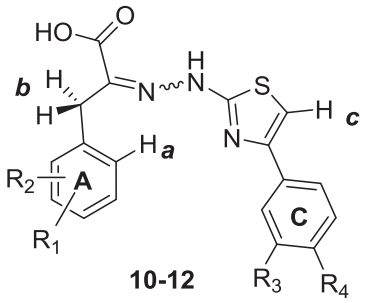
(*Z*)-2-(2-(5-(3,4-dichlorophenyl)thiazol-2-yl) hydrazono)-3-(2-nitrophenyl)propanoic acid

Fig. 2. 4EGI-1, *E*- and *Z*-isomers structures.



Scheme 3. Putative mechanism for *E*-to *Z*-isomerization around the hydrazono group in the thiazol-2-yl hydrazones.

Table 1
Comparison of the retention times in the RP-HPLC and chemical shifts of the protons and carbon distinctive to the *E*- and *Z*-isomers of the thiazol-2-yl hydrazones obtained under identical experimental conditions.



10-12

Compound	R_1	R_2	R_3	R_4	δ (ppm)				HPLC	
					H_a	H_b	H_c	C_b	R_T (min)	Ratio‡
[*E*]-**4EGI-1**	H	*o*-NO_2	Cl	Cl	7.10	4.34	7.63	29.97	8.68[&]	33%
[*Z*]-**4EGI-1**	H	*o*-NO_2	Cl	Cl	7.60	4.16	7.54	36.87	13.69[&]	67%
[*E*]-**12j**	H	*o*-NO_2	H	N_3	7.09	4.30	7.39	34.67	11.72[*]	23%
[*Z*]-**12j**	H	*o*-NO_2	H	N_3	7.54	4.20	7.36	41.50	13.53[*]	77%
[*E*]-**10d**	*p*-OH	H	Cl	Cl	7.63	3.94	7.63	30.78	16.63[#]	61%
[*Z*]-**10d**	*p*-OH	H	Cl	Cl	7.66	3.62	7.57	38.96	18.74[#]	39%
[*E*]-**11a**	*p*-NH_2	*m*-NO_2	Cl	Cl	6.46	3.90	7.43	36.49	12.30[*]	20%
[*Z*]-**11a**	*p*-NH_2	*m*-NO_2	Cl	Cl	7.30	3.66	7.63	38.24	15.09[*]	80%
[*E*]-**11b**	*p*-CO_2H	*o*-NO_2	Cl	Cl	7.21	4.34	7.65	30.32	11.81[*]	47%
[*Z*]-**11b**	*p*-CO_2H	*o*-NO_2	Cl	Cl	7.58	4.24	7.50	37.07	12.48[*]	53%
[*E*]-**12a**	H	*o*-NO_2	H	H	7.07	4.31	7.43	30.19	15.80[#]	45%
[*Z*]-**12a**	H	*o*-NO_2	H	H	7.56	4.18	7.33	36.85	16.98[#]	55%

HPLC conditions: [#]0–100% (ACN/Water/0.1%TFA) in 25 min, [*]30–100% (ACN/Water/0.1%TFA) in 25 min, [&]50–100% (ACN/Water/0.1%TFA) in 25 min. [‡]measured by HPLC in the reaction mixture.

substituents (**10c**, and **10e**–**10h**) resulted in analogues that were devoid of binding affinity. Moreover, substitution with *p*-OH or *p*-CF_3 (**10d** and **10i**, respectively) results in a 30–40% loss in binding affinity. Evidently, we cannot suggest any correlation between binding affinity and physicochemical properties such as ClogP and number of H-bond donors or acceptors (Table 2).

Table 2
4EGI-1-derived thiazol-2-yl hydrazones **10** with mono-substitution on ring **A**.

Entry	Compound	R_1	R_2	R_3	R_4	FP ratio[a]	CLogP[c]	No. of H-donors	No. of H-acceptors
1	[*Z*]-**4EGI-1**	*o*-NO_2	H	Cl	Cl	1.0	4.755	2	6
2	[*E*]-**4EGI-1**	*o*-NO_2	H	Cl	Cl	0.9	4.755	2	6
3	[*Z*]-**10a**	*m*-NO_2	H	Cl	Cl	0.9	4.835	2	6
4	[*E*]-**10a**	*m*-NO_2	H	Cl	Cl	0.8	4.835	2	6
5	[*Z*]-**10b**	*p*-NO_2	H	Cl	Cl	1.0	4.835	2	6
6	[*E*]-**10b**	*p*-NO_2	H	Cl	Cl	0.8	4.835	2	6
7	[*Z*]-**10c**	H	H	Cl	Cl	0.4	5.092	2	4
8	[*E*]-**10c**	H	H	Cl	Cl	0.4	5.092	2	4
9	[*Z*]-**10d**	*p*-OH	H	Cl	Cl	0.7	4.425	3	5
10	[*E*]-**10d**	*p*-OH	H	Cl	Cl	0.6	4.425	3	5
11	[*Z*]-**10e**	*p*-CO_2H	H	Cl	Cl	NA[b]	4.835	3	6
12	[*E*]-**10e**	*p*-CO_2H	H	Cl	Cl	NA[b]	4.835	3	6
13	[*Z*]-**10f**	*p*-NH_2	H	Cl	Cl	NA[b]	3.865	3	5
14	[*E*]-**10f**	*p*-NH_2	H	Cl	Cl	NA[b]	3.865	3	5
15	[*Z*]-**10g**	*p*-Cl	H	Cl	Cl	NA[b]	5.805	2	4
16	[*E*]-**10g**	*p*-Cl	H	Cl	Cl	NA[b]	5.805	2	4
17	[*Z*]-**10h**	*p*-F	H	Cl	Cl	NA[b]	5.235	2	5
18	[*E*]-**10h**	*p*-F	H	Cl	Cl	NA[b]	5.235	2	5
19	[*Z*]-**10i**	*p*-CF_3	H	Cl	Cl	0.7	5.975	2	7
20	[*E*]-**10i**	*p*-CF_3	H	Cl	Cl	0.6	5.975	2	7

[a] IC_{50} of [*Z*]-**4EGI-1**/IC_{50} of compound in the same plate. IC_{50} of [*Z*]-**4EGI-1** in the FP assay ranged between 42 and 47 μM.
[b] NA: not active.
[c] Chemdraw predicted value of *n*-octanol/water partition coefficient.

Table 3
4EGI-1-derived thiazol-2-yl hydrazones **11** with di-substitution on ring **A**.

Entry	Compound	R_1	R_2	R_3	R_4	FP ratio[a]	CLogP[b]	No. of H-donors	No. of H-acceptors
1	[*Z*]-**11a**	*m*-NO_2	*p*-NH_2	Cl	Cl	3.0	4.748	3	7
2	[*E*]-**11a**	*m*-NO_2	*p*-NH_2	Cl	Cl	1.0	4.748	3	7
3	[*Z*]-**11b**	*o*-NO_2	*p*-CO_2H	Cl	Cl	0.9	4.708	3	8
4	[*E*]-**11b**	*o*-NO_2	*p*-CO_2H	Cl	Cl	0.6	4.708	3	8
5	[*Z*]-**11c**	*m*-NO_2	*p*-OH	Cl	Cl	1.1	4.804	3	7
6	[*E*]-**11c**	*m*-NO_2	*p*-OH	Cl	Cl	0.9	4.804	3	7
7	[*Z*]-**11d**	*m*-NO_2	*p*-OMe	Cl	Cl	0.4	4.974	2	7
8	[*E*]-**11d**	*m*-NO_2	*p*-OMe	Cl	Cl	0.4	4.974	2	7
9	[*E*]-**11e**	*o*-NO_2	*p*-Br	Cl	Cl	1.1	5.617	2	6
10	[*E*]-**11e**	*o*-NO_2	*p*-Br	Cl	Cl	1.0	5.617	2	6
11	[*Z*]-**11f**	*m*-NO_2	*p*-Cl	Cl	Cl	0.6	5.398	2	6
12	[*E*]-**11f**	*m*-NO_2	*p*-Cl	Cl	Cl	0.3	5.398	2	6
13	[*Z*]-**11g**	*m*-NO_2	*p*-F	Cl	Cl	0.3	4.678	2	7
14	[*E*]-**11g**	*m*-NO_2	*p*-F	Cl	Cl	0.3	4.678	2	7

[a] IC_{50} of [*Z*]-**4EGI-1**/IC_{50} of compound in the same plate. IC_{50} of [*Z*]-**4EGI-1** in the FP assay ranged between 42 and 47 μM.
[b] Chemdraw predicted value of *n*-octanol/water partition coefficient.

Examination of ring **A** di-substituted **4EGI-1**-derivatives (**11**, Table 3) underscores the significance of the nitro substituent. Mono-substituted derivatives with groups other than nitro that do not bind to eIF4E regain binding affinity upon addition of a nitro group (cf. **10e** and **11b** – *p*-CO_2H and *p*-CO_2H, *o*-NO_2, respectively; **10f** and **11a** – *p*-NH_2 and *p*-NH_2, *m*-NO_2, respectively; and **10g** and **11f** – *p*-Cl and *p*-Cl, *m*-NO_2, respectively). The derivative with the highest binding affinity to eIF4E in this series was **11a**, which is comprised of the 3-(*m*-nitro, *p*-aminophenyl)propionic acid moiety. Not only that the isomer [*Z*]-**11a** was 3-fold more potent than the [Z]-**4EGI-1**, but it was also 3-fold more potent than the corresponding *E*-isomer [*E*]-**11a**, and as such displays the largest difference in binding affinity between two isomers of the same **4EGI-1** derivative. Like in the series of ring **A** mono-substituted analogues of **4EGI-1** we do not observe any correlation between binding affinity and CLogP or the number of hydrogen bond acceptors or donors in the molecule.

The **4EGI-1** analogs that maintain *o*-nitro-substituent on ring **A** and are only mono-substituted on ring C reveal some interesting structure-binding affinity relationships (analogs **12** in Table 4). Elimination of the 3,4-dichloro substitution from ring **C** leads to a significant loss in binding affinity (cf. **12a** with **4EGI-1**, Tables 4 and 2, respectively). Nevertheless, introduction of a *p*-Br substituent as in **12k** does not contribute much to the affinity relative to the non-substituted **12a**. Importantly, introduction of a non-charged polar hydroxyl group, **12b**, negative polar carboxyl group, **12c**, positive charged polar amino group, **12d**, or *N*-morpholino group, **12e** resulted in analogues with significantly lower binding affinity to eIF4E than the parent **4EGI-1**. Interestingly, ring **C** tolerates substitution by the *N*-pyrolidine moiety, **12f**, the *p*-N_3 group, **12j**, and the hydrophobic substituents *p*-cyclohexyl, *p*-phenyl, and *p*-biphenyl (**12g**, **12h**, and **12i**, respectively).

The divergence in the structure–binding affinity relationships of the phenyl rings **A** and **C** suggest that the binding sub-sites on eIF4E for the phenyl rings **A** and **C** in **4EGI-1** and its analogues differ in their nature. While the sub-site interacting with ring **A** accommodates polar and hydrophilic groups such as NO_2 and NH_2, the sub-site interacting with ring **C** has preference for hydrophobic moieties and is quite relaxed in accommodating large substituents such as cyclohexyl and biphenyl.

2.5. Intrinsic tryptophan fluorescence intensity quenching assay – validation

The relative abundance of tryptophan residues in eIF4E provided the opportunity to have an independent method to assess

Table 4
4EGI-1-derived thiazol-2-yl hydrazones modified on ring **C**, **12a**–**k**, and its eIF4E/eIF4G interaction inhibitory activities.

Entry	Compound	R_1	R_2	R_3	R_4	FP ratio[a]	CLogP[d]	No. of H-donors	No. of H-acceptors
1	[*Z*]-**12a**	H	*o*-NO_2	H	H	0.5	3.440	2	6
2	[*E*]-**12a**	H	*o*-NO_2	H	H	0.4	3.440	2	6
3	[*Z*]-**12b**	H	*o*-NO_2	H	*p*-OH	NA[b]	2.979	3	7
4	[*E*]-**12b**	H	*o*-NO_2	H	*p*-OH	NA[b]	2.979	3	7
5	[*Z*]-**12c**	H	*o*-NO_2	H	*p*-CO_2H	0.4	3.259	3	8
6	[*E*]-**12c**	H	*o*-NO_2	H	*p*-CO_2H	0.3	3.259	3	8
7	[*Z*]-**12d**	H	*o*-NO_2	H	*p*-NH_2	0.4	2.407	3	7
8	[*E*]-**12d**	H	*o*-NO_2	H	*p*-NH_2	NA[b]	2.407	3	7
9	[*Z*]-**12e**	H	*o*-NO_2	H	*p*-Morpholino	0.6	3.014	2	8
10	[*Z*]-**12e**	H	*o*-NO_2	H	*p*-Morpholino	0.4	3.014	2	8
11	[*Z*/*E*]-**12f**	H	*o*-NO_2	H	*p*-Pyrolidino	1.1	3.837	2	7
12	[*Z*]-**12g**	H	*o*-NO_2	H	*p*-Cy-hexyl	0.8	6.060	2	6
13	[*E*]-**12g**	H	*o*-NO_2	H	*p*-Cy-hexyl	NA[b]	6.060	2	6
14	[*Z*]-**12h**[c]	H	*o*-NO_2	H	*p*-Ph	1.2	5.328	2	6
15	[*Z*]-**12i**	H	*o*-NO_2	H	*p*-*Ph*-Ph	0.9	4.614	2	6
16	[*E*]-**12i**	H	*o*-NO_2	H	*p*-*Ph*-Ph	0.4	4.614	2	6
17	[*Z*]-**12j**	H	*o*-NO_2	H	*p*-N_3	1.0	4.038	2	8
18	[*E*]-**12j**	H	*o*-NO_2	H	*p*-N_3	1.0	4.038	2	8
19	[*Z*]-**12k**	H	*o*-NO_2	H	*p*-Br	0.6	4.310	2	6
20	[*E*]-**12k**	H	*o*-NO_2	H	*p*-Br	0.5	4.310	2	6

[a] IC_{50} of [*Z*]-**4EGI-1**/IC_{50} of compound in the same plate. IC_{50} of [*Z*]-**4EGI-1** in the FP assay ranged between 42 and 47 μM.
[b] NA: not active.
[c] [*E*]-**12h** was not obtained.
[d] Chemdraw predicted value of n-octanol/water partition coefficient.

small molecule-eIF4E interactions. Measurement of the change in intrinsic fluorescence intensity of eIF4E, which is sensitive to the local environment, as a function of ligand concentration will report on the K_d for the ligand of interest [23]. We were very pleased to see that the K_d for the parent [*Z*]-**4EGI-1**, [*Z*]-**11a**, and [*Z*]-**12b** (K_d = 8.74, 5.01, and 36.6 mM, respectively) follows closely the relative binding affinities as measured in the FP assay ((IC_{50} ([*Z*]-**4EGI-1**)/IC_{50}) (**4EGI-1** derivative)) = 1.0, 3.0, and not active, respectively, Fig. 3). While FP assay is a competitive binding assay and reports on a competitive ligand displacement phenomenon the dose dependent change in intrinsic fluorescent intensity reports on a global change induce by the binding of the small molecule to the protein and is less informative on the site of the bimolecular interaction. The very good correlation between the two measurement methods strongly supports the involvement of the small molecule in disrupting a PPI. Evidently, competitive binding of the small molecule displacing the fluoresceinyl-labeled eIF4G-derived peptide as measured by FP is tightly linked to global structural changes in the target protein as reflected by the change in intrinsic fluorescence induced by the direct small ligand-eIF4E interaction.

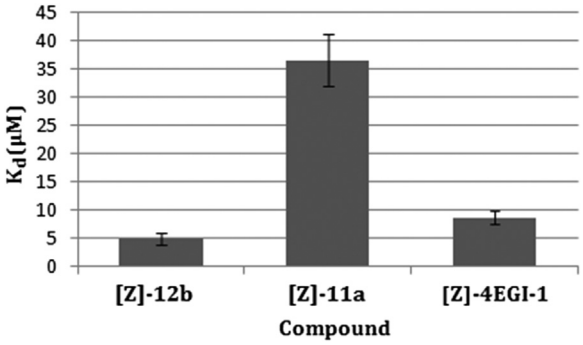


Fig. 3. K_d's for [*Z*]-4EGI-1 and representative analogues. K_d's were calculated from the eIF4E intrinsic tryptophan fluorescence assay.

2.6. Translational down-regulation of regulatory proteins

Since expression of many regulatory proteins is translationally regulated and therefore highly dependent on the activity of translation initiation factors, we tested selected **4EGI-1** analogues; [*Z*]-**11a** and [*Z*]-**12b**, a highly potent and a non-active compound, respectively, and the parent compound [*Z*]-**4EGI-1** for their effects on expression of regulatory proteins as well as housekeeping proteins (Fig. 4). As anticipated, in this cell-based assay the two compounds that bind avidly to eIF4E, i.e. [*Z*]-**4EGI-1** and [*Z*]-**11a** markedly reduced the expressions of the regulatory proteins; cyclin D1, cyclin E, and Survivin, while the expressions of housekeeping proteins such as β-Actin and α-Tubulin were not affected. That was not the case for the non-active derivative; [*Z*]-**12b** which had no effect on the expression of the evaluated proteins. Down-regulation of most regulatory proteins was likely translational because the compounds had minimal effects on the levels of the respective mRNAs (Fig. 5). These findings are consistent with our previous observation that **4EGI-1** preferentially affect the expression of regulatory proteins [8]. Of note was the significant reduction in cyclin D1 expression, which is reported to be essential for Ras-Raf-MEK driven cell proliferation, such as the CRL-2813 melanoma cancer cells that harbor activating mutation of Raf gene [24]. The lesser effect of the active compounds [Z]-**4EGI-1** and [Z]-**11a** had on cyclin E expression as compared to their effect on cyclin D1 and survivin may be due to the greater stability of cyclin E (cf. life time ~5 h [25] for cyclin E, compared to ~ 30 min for cyclin D1) [26].

2.7. Disruption of eIF4E/eIF4G interaction

To determine directly the disruption of eIF4E/eIF4G interaction by these analogues we tested [*Z*]-**11a**, the **4EGI-1** analog with the highest binding affinity reported in this study, in intact cancer CRL-2813 cells (Fig. 6). In this experiment, lysates of cells treated with either [*Z*]-**11a** or DMSO were loaded on m^7GTP affinity column to separate the unbound eIF4G in the flow-through (not shown) from the eIF4E, and eIF4E–eIF4G and eIF4E–4E-BP1 complexes in the retentate. Western blot analysis of the column retentate

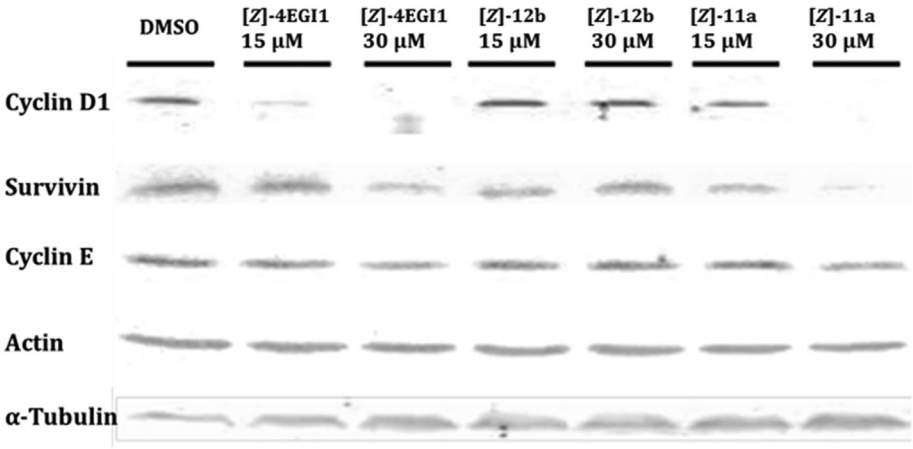


Fig. 4. Effect of [*Z*]-4EGI-1 and analogues on regulatory and house-keeping protein expression. Human CRL-2813 melanoma cells were treated with the indicated concentrations of each compound for 6 h. Expression of the indicated proteins was determined by Western blot analysis. A representative gel from three independent experiments is shown.

demonstrated decrease in the amount of eIF4E/eIF4G complex and increase in 4E-BP1 in the cells incubated with [*Z*]**-11a** relative to control. These results confirm the conclusions drawn from the FP and Intrinsic tryptophan fluorescence intensity quenching assays, as well as the down-regulation of regulatory proteins experiment. Moreover, they are consistent with our previously reported observation that **4EGI-1** inhibits eIF4E/eIF4G interaction independently of the 4E-BPs and may indeed cause a significant enhancement of 4E-BP1 binding to eIF4E [8].

3. Conclusions

Protein–Protein interactions (PPIs) coordinate and control many cellular processes, such as chemotaxis, proliferation, differentiation, endocytosis and apoptosis. The flat, large, and feature-poor protein–protein interface makes targeting PPIs by small molecule inhibitors a challenging task. Our current molecular probe optimization efforts of **4EGI-1**, the prototypical small molecule eIF4E/eIF4G protein–protein interaction inhibitor, provide important insight that will be instrumental, in the future, for further enhancement of its potency and improvement of its target selectivity.

Our SAR analysis elucidated the nature of the **4EGI-1**/eIF4E binding site and the structural latitude of the **4EGI-1** related scaffold. This insight is instrumental in guiding our hit-to-lead optimization to generate powerful and selective molecular probes for use in animal models of diseases associated with unregulated cap-dependent translation. Our findings suggest that the phenyl rings **A** and **C** differ in their preferred substituents. While ring **A** best accommodates a combination of polar substituents such as *o*-nitro and *p*-amino groups as in **11a**, the preferred substituents on ring **C** are hydrophobic substituent such as *p*-phenyl as in the potent **12h**. In addition, our better understanding of the plausible role the *E*- and *Z*-isomers around the hydrazono function in the **4EGI-1** scaffold affect physicochemical properties will be very helpful in guiding our medicinal chemistry efforts. Moreover, the observed correlation between cell-free and cell-based target specific assays is very encouraging and promises effective reduction in off-target activities.

4. Experimental

4.1. Chemistry

All reagents and solvents were purchased from commercial sources and used without further purification. All the reaction

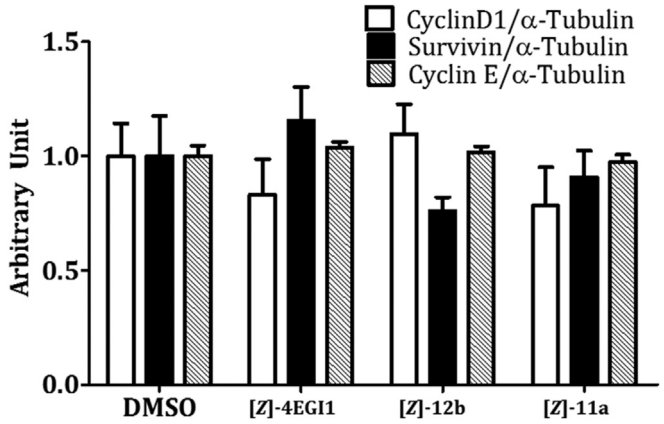


Fig. 5. Effect of [*Z*]-4EGI-1 and analogues on mRNA levels of cyclin D1, cyclin E, survivin, α-tubulin, and β-actin. CRL-2813 cells were incubated with 30 μM of each compound for 6 h. Expression of cyclin D1, cyclin E, surviving, and α-tubulin was determined by real time PCR analysis. Each experiment was carried out in triplicate, data shown are mean of three independent experiments, and error bars are ±S.E.M.

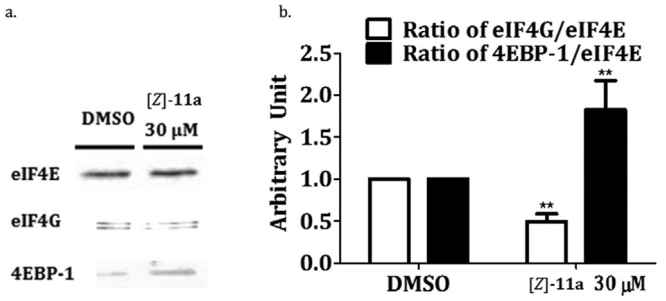


Fig. 6. Disruption of eIF4E/eIF4G interaction in CRL-2813 melanoma cancer cells by the [*Z*]-11a, a highly potent analogue of 4EGI-1. CRL-2813 cells were incubated with either vehicle (DMSO) or 30 μM of [*Z*]**-11a** for 6 h and lysed. The lysate was separated on cap-affinity column and the retentate was analyzed SDS-PAGE. Proteins were detected by Western blot analysis. **a**. A representative gel from three independent experiments is shown. Retentates obtained from lysates of cells incubated for 6 h with either [*Z*]**-11a** (right lane) or vehicle (DMSO, left lane), which were run side-by-side. **b**. Quantitative analysis of Western blots such as (**a**) obtained in three identical and independent pull-down experiments. Data shown are mean of three independent experiments, and error bars are ±S.E.M.

solvents were anhydrous and the reactions were maintained under inert atmosphere. Melting points were determined using a Mel-Temp Electrothermal apparatus and were uncorrected. Proton and carbon NMR analyses were performed on Varian 400 MHz and 500 MHz spectrometers using DMSO-d_6, acetone-d_6 and $CDCl_3$ as solvents. Chemical shifts (δ) are reported in ppm relative to TMS with solvent residual peaks as internal standard. All the reactions were monitored by LC–MS analysis on reverse phase (column Waters Symmetry C18, 2.1 × 100 mm, 3.5 μm particle size) using a Waters Alliance 2695/Micromass ZQ with the binary system water/acetonitrile containing 0.1% of formic acid as eluent. Purifications by flash chromatography were performed on Biotage SP1 using silica gel pre-packed columns and were monitored by UV at 254 and 280 nm. The HR-MS analyses were performed at the FAS Center for Systems Biology, Harvard University. The purity of all final compounds was determined by analytical HPLC on reverse phase (column XBridge BEH130 C18, 4.6 × 100 mm, 5 μm particle size) using a Waters Alliance 2695 with the binary system water/acetonitrile containing 0.1% of trifluoracetic acid (TFA) as eluent. In the absence of signals corresponding to the $CO_2\underline{H}$ and N$\underline{H}$ in the ^{1}H NMR the presence of the carboxyl was confirmed by ^{13}C NMR and LCMS and that of NH by HRMS.

4.2. General procedure-1: synthesis of the di-substituted benzaldehydes, **1**

Method A. 10 mmol of the desired *p*-substituted benzaldehyde was dissolved in 10 mL of anhydrous acetonitrile and cooled to 0 °C, then 1.01 equiv. of nitronium tetrafluoroborate, NO_2BF_4, were added under vigorous stirring. The cold bath was then removed and the reaction was stirred for 3 h at room temperature. The solvent was partially evaporated then poured into 50 mL of ice-cold water. The product was obtained as a precipitate, which was filtered and washed with cold water. The product was purified using reverse phase column chromatography with gradient increase of methanol in water containing 0.1% of formic acid as eluent.

Method B. 10 mmol of the desired *p*-substituted benzaldehyde was dissolved in 5 mL of conc. sulfuric acid, and cooled to 0 °C, and then 1.2 equiv. of nitric acid was dissolved in 1 mL of conc. sulfuric acid, and then slowly added to the reaction mixture. The reaction was allowed to warm gradually to room temperature and stirred for 30 min at room temperature. It was then poured into 50 mL of ice-cold water. The produced precipitate was filtered and washed with cold water. The product was purified on reverse phase column chromatography with gradient increase of methanol in water containing 0.1% of formic acid as eluent.

4.2.1. N-(4-Formyl-2-nitrophenyl)acetamide, **1a**

Using general procedure-1, method A, employing *p*-acetamidobenzaldehyde to afford **1a** as a yellow solid, 71% (1.47 g) yield, mp 175 °C; RP-HPLC (C18): 0–70% (ACN/DDW/0.1% TFA) in 25 min, t_R = 10.92 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.61 (s, 1H), 9.98 (s, 1H), 8.45 (d, *J* = 1.9 Hz, 1H), 8.15 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.88 (d, *J* = 8.5 Hz, 1H), 2.11 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 191.64, 170.08, 142.29, 136.64, 134.21, 132.71, 127.49, 125.61, 24.20; HRMS (ESI) for $C_9H_8N_2O_4$ $[M-H]^-$: *m*/*z* calcd; 207.04841, found; 207.04124.

4.2.2. 4-Formyl-3-nitrobenzoic acid, **1b**

Using general procedure-1, method B, employing 4-formylbenzoic acid to afford **1b** as a white solid, 80% (1.56 g) yield, mp 192 °C; RP-HPLC (C18): 0–50% (ACN/DDW/0.1% TFA) in 25 min, t_R = 9.23 min, purity of 100%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.09 (s, 1H), 8.45 (s, 1H), 8.26 (d, *J* = 7.8 Hz, 1H), 8.01 (d, *J* = 7.8 Hz, 1H). ^{13}C NMR (100 MHz, DMSO) δ 191.83, 166.23, 148.83, 138.90, 134.05, 132.89, 131.34, 125.08; LCMS (ESI) for $C_8H_5NO_5$ $[M-H]^-$: *m*/*z* calcd; 194.02, found; 193.89.

4.2.3. 4-Chloro-3-nitrobenzaldehyde, **1c**

Using general procedure-1, method B, employing 4-chlorobenzaldehyde to afford **1c** as a white solid, 94% (1.74 g) yield, mp 50 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 13.26 min, purity of 99%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.04 (s, 1H), 8.54 (s, 1H), 8.16 (d, *J* = 8.3 Hz, 1H), 7.98 (dd, *J* = 8.2, 2.5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ 191.37, 148.62, 136.31, 134.17, 133.44, 131.30, 126.90; LCMS (ESI) for $C_7H_4ClNO_3$ $[M-H]^-$: *m*/*z* calcd; 183.99, found; 183.99.

4.2.4. 4-Fluoro-3-nitrobenzaldehyde, **1d**

Using general procedure-1, method B, employing 4-fluorobenzaldehyde to afford **1d** as a pale yellow solid, 79% (1.33 g) yield, mp 30 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.29 min, purity of 96%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.04 (d, *J* = 1.8 Hz, 1H), 8.64 (dt, *J* = 7.5, 2.1 Hz, 1H), 8.38–8.17 (m, 1H), 7.79 (t, *J* = 9.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ 191.21, 159.83, 157.14, 137.05, 136.94, 133.55, 128.36, 120.52, 120.30; LCMS (ESI) for $C_7H_4FNO_3$ $[M-H]^-$: *m*/*z* calcd; 168.02, found; 168.00.

4.2.5. 4-Methoxy-2-nitrobenzaldehyde, **1e**

Using general procedure-1, method B, employing 4-methoxybenzaldehyde to afford **1e** as a yellow solid, 89% (1.62 g) yield, mp 79 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.40 min, purity of 100%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.95 (s, 1H), 8.43 (d, *J* = 2.1 Hz, 1H), 8.20 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.58 (d, *J* = 8.8 Hz, 1H), 4.04 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 191.13, 156.91, 135.68, 129.46, 127.13, 115.69, 110.91, 58.16; LCMS (ESI) for $C_8H_7NO_4$ $[M+H]^+$: *m*/*z* calcd; 182.14, found; 181.94.

4.3. General procedure-2: synthesis of the oxazoles **2**, or acrylic acids, **3**

5 mmol of the substituted benzaldehyde was dissolved in 20 mL of acetic anhydride then 1.2 equiv. of *N*-acetyl glycine and 5 equiv. of sodium acetate were added and the reaction was stirred under reflux for 4 h. Then solvent was partially evaporated then was poured into 50 mL of crushed ice and stirred vigorously for 30 min. A precipitate was obtained which was separated and washed with cold water and dried under vacuum to get the oxazolyl product **2**, or its hydrolyzed acrylic acid, **3**.

4.3.1. N-(4-((2-Methyl-5-oxooxazol-4(5H)-ylidene)methyl)phenyl) acetamide, **2a**

Using general procedure-2, employing *p*-acetaminobenzaldehyde to afford **2a** as a yellow solid, 67% (0.82 g) yield, mp 235 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.25 (s, 1H), 8.10 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 8.7 Hz, 2H), 7.12 (s, 1H), 2.36 (s, 3H), 2.06 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 169.52, 168.22, 166.38, 142.61, 133.80, 131.45, 130.45, 128.37, 119.37, 24.85, 16.02; HRMS (ESI) for $C_{13}H_{12}N_2O_3$ $[M+H]^+$: *m*/*z* calcd; 245.0848, found; 245.09310.

4.3.2. 4-(4-Fluorobenzylidene)-2-methyloxazol-5(4H)-one, **2b**

Using general procedure-2, employing 4-fluorobenzaldehyde to afford **2b** as a yellow solid, 56% (1.16 g) yield, mp 150 °C; RP-HPLC (C18): 0–70% (ACN/DDW/0.1% TFA) in 25 min, t_R = 12.99 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.21 (t, *J* = 8.0 Hz, 2H), 7.21–7.32 (m, 2H), 7.20 (s, 1H), 2.36 (s, 3H); ^{13}C NMR (DMSO, 100 MHz): δ 167.48, 151.92, 145.03, 135.08, 132.87, 130.18, 129.24, 116.83, 116.62, 115.02, 16.02; ^{19}F NMR (DMSO-d_6, 375.78 MHz): δ −108.25; LCMS (ESI) for $C_{11}H_8FNO_2$ $[M+H]^+$: *m*/*z* calcd; 206.05, found; 206.00.

4.3.3. 4-((2-Methyl-5-oxooxazol-4(5H)-ylidene)methyl)-3-nitrobenzoic acid, **2c**

Using general procedure-2, employing **1b** to afford **2c** as a white solid, 90% (1.24 g) yield, mp 236 °C (dec.); RP-HPLC (C18): 0–30% (ACN/DDW/0.1% TFA) in 25 min, t_R = 10.25 min, purity of 99%; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.10 (bs, 1H), 8.64 (s, 1H), 8.26 (d, *J* = 9.6 Hz, 1H), 8.05 (s, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 2.40 (s, 3H); HRMS (ESI) for $C_{12}H_8N_2O_6$ $[M-H]^-$: *m*/*z* calcd; 275.0310, found; 275.0291.

4.3.4. 4-(4-Hydroxy-3-nitrobenzylidene)-2-methyloxazol-5(4H)-one, **2d**

Using general procedure-2, employing 4-hydroxy-3-nitrobenzaldehyde to afford **2d** as a yellow solid, 95% (1.18 g) yield, mp 164 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 10.02 min, purity of 91%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.91 (d, *J* = 2.1 Hz, 1H), 8.48 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.55 (d, *J* = 8.5 Hz, 1H), 7.31 (s, 1H), 2.34 (s, 3H), (OH was not detected); ^{13}C NMR (100 MHz, DMSO) δ 169.04, 169.00, 167.48, 144.78, 142.25, 138.56, 135.23, 132.74, 128.79, 126.54, 16.18; HRMS (ESI) for $C_{11}H_8N_2O_5$ $[M+H]^+$: *m*/*z* calcd; 249.05060, found; 249.05150.

4.3.5. 4-(4-Chloro-3-nitrobenzylidene)-2-methyloxazol-5(4H)-one, **2e**

Using general procedure-2, employing **1c** to afford **2e** as a pale yellow solid, 80% (1.07 g) yield, mp 172 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 16.79 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.80 (d, *J* = 2.3 Hz, 1H), 8.42 (dd, *J* = 8.6, 2.0 Hz, 1H), 7.89 (d, *J* = 8.5 Hz, 1H), 7.30 (s, 1H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 169.34, 167.40, 148.28, 136.84, 135.78, 134.15, 133.02, 128.55, 127.41, 126.01, 16.24. LCMS (ESI) for $C_{11}H_7ClN_2O_4$ $[M+H]^+$: *m*/*z* calcd; 267.01, found; 267.07.

4.3.6. 4-(4-Fluoro-3-nitrobenzylidene)-2-methyloxazol-5(4H)-one, **2f**

Using general procedure-2, employing **1d** to afford **2f** as a yellow solid, 82% (1.03 g) yield, mp 150 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 15.53 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.99 (dt, *J* = 7.6, 1.8 Hz, 1H), 8.52 (ddt, *J* = 8.3, 3.7, 1.7 Hz, 1H), 7.71 (ddd, *J* = 11.5, 8.7, 1.2 Hz, 1H), 7.34 (s, 1H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 169.01, 167.51, 157.36, 154.69, 134.98, 131.10, 129.55, 126.42, 120.08, 119.87, 16.22. LCMS (ESI) for $C_{11}H_7FN_2O_4$ $[M+H]^+$: *m*/*z* calcd; 251.04, found; 251.14.

4.3.7. 4-(4-Methoxy-3-nitrobenzylidene)-2-methyloxazol-5(4H)-one, **2g**

Using general procedure-2, employing **1e** to afford **2g** as a brown oil, 77% (1.01 g) yield; RP-HPLC (C18): 0–70% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.07 min, purity of 97%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.73 (s, 1H), 8.39 (d, *J* = 8.6 Hz, 1H), 7.49 (d, *J* = 8.9 Hz, 1H), 7.25 (s, 1H), 3.98 (s, 3H), 2.38 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 167.75, 154.23, 139.92, 138.54, 133.13, 128.52, 127.88, 126.32, 115.55, 57.81, 16.13; HRMS (ESI) for $C_{12}H_{10}N_2O_5$ $[M+H]^+$: *m*/*z* calcd; 263.06, found; 263.01.

4.3.8. 4-((2-Methyl-5-oxooxazol-4(5H)-ylidene)methyl)benzoic acid, **2h**

Using general procedure-2, employing 4-formylbenzoic acid to afford **2h** as a yellow solid, 98% (1.13 g) yield, mp 118 °C; RP-HPLC (C18): 0–50% (ACN/DDW/0.1% TFA) in 25 min, t_R = 13.31 min, purity of 95%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.72 (bs, 1H), 8.26 (d, *J* = 6.3 Hz, 2H), 8.02 (d, *J* = 6.2 Hz, 2H), 7.26 (s, 1H), 2.41 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 168.58, 167.82, 167.45, 137.67, 134.99, 132.83, 132.49, 130.28, 128.71, 16.17; HRMS (ESI) for $C_{12}H_9NO_4$ $[M+H]^+$: *m*/*z* calcd; 232.06043, found; 232.06007.

4.3.9. N-(4-((2-methyl-5-oxooxazol-4(5H)-ylidene)methyl)-2-nitrophenyl)acetamide, **2i**

Using general procedure-2, employing **1a** to afford **2i** as a yellow solid, 80% (1.16 g) yield, mp 205 °C; RP-HPLC (C18): 0–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 13.53 min, purity of 95%; ^{1}H NMR (399 MHz, Chloroform-*d*) δ 10.49 (s, 1H), 9.00 (d, *J* = 1.9 Hz, 1H), 8.89 (d, *J* = 8.9 Hz, 1H), 8.33 (dd, *J* = 8.9, 2.0 Hz, 1H), 7.06 (s, 1H), 2.44 (s, 3H), 2.33 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.32, 160.73, 138.93, 136.43, 134.05, 129.36, 128.54, 127.65, 122.25, 26.02, 16.04; HRMS (ESI) for $C_{13}H_{11}N_3O_5$ $[M+H]^+$: *m*/*z* calcd; 290.07715, found; 290.07583.

4.3.10. 2-Acetamido-3-(3-nitrophenyl)acrylic acid, **3a**

Using general procedure-2, employing 3-nitrobenzaldehyde to afford **3a** as a yellow solid, 92% (1.15 g) yield, mp 192 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 15.24 min, purity of 96%. ^{1}H NMR (399 MHz, DMSO-d_6) δ 9.63 (s, 1H), 8.38 (s, 1H), 8.15 (d, *J* = 8.1 Hz, 1H), 7.96 (d, *J* = 6.7 Hz, 1H), 7.66 (td, *J* = 8.1, 3.2 Hz, 1H), 7.30 (d, *J* = 3.7 Hz, 1H), 1.99 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.61, 166.80, 148.45, 136.56, 130.59, 130.27, 128.01, 124.19, 123.79, 23.31; LCMS (ESI) for $C_{11}H_{10}N_2O_5$ $[M-H]^-$: *m*/*z* calcd; 249.09, found; 249.06.

4.3.11. 2-Acetamido-3-(4-nitrophenyl)acrylic acid, **3b**

Using general procedure-2, employing 4-nitrobenzaldehyde to afford **3b** as a yellow solid, 86% (1.00 g) yield, mp 206 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 9.24 min, purity of 96%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 9.68 (s, 1H), 8.19 (d, *J* = 8.5 Hz, 2H), 7.79 (d, *J* = 8.6 Hz, 2H), 7.21 (s, 1H), 1.99 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.82, 166.69, 147.39, 141.52, 131.15, 131.01, 124.47, 124.17, 23.31; LCMS (ESI) for $C_{11}H_{10}N_2O_5$ $[M-H]^-$: *m*/*z* calcd; 249.09, found; 249.02.

4.3.12. 2-Acetamido-3-(4-chlorophenyl)acrylic acid, **3c**

Using general procedure-2, employing 4-chlorobenzaldehyde to afford **3c** as a yellow solid, 98% (1.18 g) yield, mp 190 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 10.58 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 9.47 (s, 1H), 7.60 (d, *J* = 8.4 Hz, 2H), 7.44 (d, *J* = 8.4 Hz, 2H), 7.17 (s, 1H), 1.96 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 169.83, 166.93, 134.16, 132.02, 130.08, 129.72, 129.23, 23.26; LCMS (ESI) for $C_{11}H_{10}ClNO_3$ $[M-H]^-$: *m*/*z* calcd; 238.03, found; 237.99.

4.3.13. 2-Acetamido-3-(4-(trifluoromethyl)phenyl)acrylic acid, **3d**

Using general procedure-2, employing 4-(trifluoromethyl) benzaldehyde to afford **3d** as a white solid, 91% (1.25 g) yield, mp 185 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.82 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.59 (s, 1H), 7.40 (q, *J* = 8.0 Hz, 4H), 7.21 (s, 1H), 1.97 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 169.31, 166.82, 138.79, 130.75, 130.22, 128.89, 128.14, 126.29, 125.94, 23.23; ^{19}F NMR (DMSO-d_6, 375.78 MHz): δ −61.70; LCMS (ESI) for $C_{12}H_{10}FNO_3$ $[M-H]^-$: *m*/*z* calcd; 272.06, found; 272.09.

4.4. General procedure-3: synthesis of the 2-hydroxyacrylic acids, **4**

2 mmol of the desired oxazolyl product, **2**, or acatamido acrylic acid, **3**, was dissolved in 10 mL of 32% $HCl_{(aq)}$ and allowed to reflux for 2 h. Then was allowed to cool to room temperature and 10 mL of DDW were added. The produced precipitate was filtrated and washed with water and dried under vacuum.

4.4.1. 2-Hydroxy-3-(3-nitrophenyl)acrylic acid, **4a**

Using general procedure-3, employing **3a** to afford **4a** as a yellow solid, 91% (380 mg) yield, mp 133 °C; RP-HPLC (C18): 0–100%

(ACN/DDW/0.1% TFA) in 25 min, t_R = 12.51 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 9.92 (bs, 1H), 8.91–8.50 (m, 1H), 8.03 (dt, J = 8.3, 2.2 Hz, 2H), 7.59 (t, J = 8.0 Hz, 1H), 6.51 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 166.46, 148.59, 144.76, 137.44, 136.09, 130.35, 123.59, 122.15, 107.61; LCMS (ESI) for $C_9H_7NO_5$ [M−H]$^-$: m/z calcd; 208.03, found; 207.98.

4.4.2. 2-Hydroxy-3-(4-nitrophenyl)acrylic acid, ***4b***

Using general procedure-3, employing **3b** to afford **4b** as a yellow solid, 96% (400 mg) yield, mp 192 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 12.59 min, purity of 99%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.14 (s, 1H), 8.17 (d, J = 8.9 Hz, 2H), 7.97 (d, J = 8.9 Hz, 2H), 6.48 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 166.36, 146.02, 145.99, 142.85, 130.41, 124.25, 107.56; LCMS (ESI) for $C_9H_7NO_5$ [M−H]$^-$: m/z calcd; 208.03, found; 207.98.

4.4.3. 4-(2-Carboxy-2-hydroxyvinyl)benzoic acid, ***4c***

Using general procedure-3, employing **3c** to afford **4c** as a white solid, 93% (387 mg) yield, mp 265 °C; RP-HPLC (C18): 0–50% (ACN/DDW/0.1% TFA) in 25 min, t_R = 12.13 min, purity of 97%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.65 (s, 1H), 7.96–7.89 (m, 2H), 7.85 (d, J = 8.7 Hz, 2H), 6.45 (s, 1H); ^{13}C NMR (126 MHz, DMSO) δ 167.81, 166.72, 144.37, 140.13, 130.14, 130.03, 129.73, 108.93; LCMS (ESI) for $C_{10}H_8O_5$ [M−H]$^-$: m/z calcd; 207.04, found; 207.06.

4.4.4. 3-(4-Aminophenyl)-2-hydroxyacrylic acid, ***4d***

Using general procedure-3, employing **2a** to afford **4d** as a white solid, 90% (322 mg) yield, mp 300 °C; RP-HPLC (C18): 0–50% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.48 min, purity of 94%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 7.82 (d, J = 7.0 Hz, 2H), 7.51 (s, 2H), 7.39 (s, 1H), 7.35 (d, J = 7.2 Hz, 2H), 7.26 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 166.71, 143.27, 135.40, 131.08, 130.84, 123.88, 108.84; LCMS (ESI) for $C_9H_9NO_3$ [M−H]$^-$: m/z calcd; 178.06, found; 177.85.

4.4.5. 3-(4-Chlorophenyl)-2-hydroxyacrylic acid, ***4e***

Using general procedure-3, employing **3c** to afford **4e** as a yellow solid, 90% (356 mg) yield, mp 183 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 14.31 min, purity of 100%; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.50 (s, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.3 Hz, 2H), 7.21 (s, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.85, 166.96, 134.18, 133.43, 132.04, 129.25, 108.89; LCMS (ESI) for $C_9H_7ClO_3$ [M−H]$^-$: m/z calcd; 197.01, found; 197.00.

4.4.6. 3-(4-Fluorophenyl)-2-hydroxyacrylic acid, ***4f***

Using general procedure-3, employing **2b** to afford **4f** as a white solid, 90% (328 mg) yield, mp 160 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 12.83 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.95 (s, 1H), 7.98 (s, 1H), 7.42 (t, J = 8.0 Hz, 2H), 7.14 (t, J = 8.0 Hz, 2H); ^{13}C NMR (100 MHz, DMSO) δ 167.07, 161.60, 137.11, 132.82, 132.72, 129.31, 129.23, 116.37, 116.15, 110.56; ^{19}F NMR (DMSO-d_6, 375.78 MHz): δ −107.28; LCMS (ESI) for $C_9H_7FO_3$ [M−H]$^-$: m/z calcd; 181.04, found; 181.15.

4.4.7. 2-Hydroxy-3-(4-(trifluoromethyl)phenyl)acrylic acid, ***4g***

Using general procedure-3, employing **3d** to afford **4g** as a white solid, 90% (418 mg) yield, mp 170 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 15.00 min, purity of 96%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.11 (s, 1H), 8.10 (d, J = 7.4 Hz, 2H), 7.96 (d, J = 8.0 Hz, 2H), 7.62 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 170.65, 140.37, 129.43, 129.11, 128.92, 128.79, 127.96, 126.81, 126.76, 123.39. LCMS (ESI) for $C_{10}H_7F_3O_3$ [M−H]$^-$: m/z calcd; 231.03, found; 231.05.

4.4.8. 3-(4-Amino-3-nitrophenyl)-2-hydroxyacrylic acid, ***4h***

Using general procedure-3, employing **2i** to afford **4h** as a red solid, 71% (320 mg), mp 190 dec.; RP-HPLC (C18): 0–50% (ACN/Water/0.1%TFA) in 25 min, t_R = 15.42 min, purity of 94%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.06 (s, 1H), 9.19 (s, 1H), 8.56 (s, 1H), 7.69 (d, J = 8.8 Hz, 1H), 7.57 (s, 2H), 6.99 (d, J = 8.8 Hz, 1H), 6.33 (s, 1H); ^{13}C NMR (126 MHz, DMSO) δ 166.90, 145.97, 141.23, 137.73, 130.80, 126.23, 123.39, 119.68, 109.26; LCMS (ESI) for $C_9H_8N_2O_5$ [M−H]$^-$: m/z calcd; 223.04, found; 223.05.

4.4.9. 4-(2-Carboxy-2-hydroxyvinyl)-3-nitrobenzoic acid, ***4i***

Using general procedure-3, employing **2c** to afford **4i** as a white solid, 95% (481 mg) yield, mp 169 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 10.95 min, purity of 90%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 13.5 (bs, 3H), 8.25 (s, 1H), 7.99 (d, J = 8.2 Hz, 1H), 7.78 (d, J = 8.1 Hz, 1H), 6.44 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 169.58, 166.29, 149.79, 146.92, 140.33, 132.88, 130.84, 125.11, 123.41, 105.69; LCMS (ESI) for $C_{10}H_7NO_7$ [M−H]$^-$: m/z calcd; 252.02, found; 251.83.

4.4.10. 2-Hydroxy-3-(4-hydroxy-3-nitrophenyl)acrylic acid, ***4j***

Using general procedure-3, employing **2d** to afford **4j** as a yellow solid, 93% (420 mg) yield, mp 211 °C (dec.); RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.74 min, purity of 89%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.40 (d, J = 2.2 Hz, 1H), 7.80 (dd, J = 8.7, 2.2 Hz, 1H), 7.54 (s, 1H), 7.44 (s, 1H), 7.34 (s, 1H), 7.23 (d, J = 8.6 Hz, 1H), 6.38 (s, 1H); ^{13}C NMR (126 MHz, DMSO) δ 166.70, 151.86, 142.39, 137.46, 136.52, 127.09, 125.68, 119.68, 108.23; LCMS (ESI) for $C_9H_7NO_6$ [M−H]$^-$: m/z calcd; 224.03, found; 223.93.

4.4.11. 3-(4-Chloro-3-nitrophenyl)-2-hydroxyacrylic acid, ***4k***

Using general procedure-3, employing **2e** to afford **4k** as a yellow solid, 88% (432 mg) yield, mp 152 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 14.12 min, purity of 100%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.02 (bs, 1H), 8.43 (d, J = 2.1 Hz, 1H), 7.98 (dd, J = 8.6, 2.1 Hz, 1H), 7.70 (d, J = 8.5 Hz, 1H), 6.46 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 166.23, 148.20, 145.28, 136.42, 134.60, 132.20, 125.73, 123.23, 106.61; LCMS (ESI) for $C_9H_6ClNO_5$ [M−H]$^-$: m/z calcd; 241.99, found; 242.08.

4.4.12. 3-(4-Fluoro-3-nitrophenyl)-2-hydroxyacrylic acid, ***4l***

Using general procedure-3, employing **2f** to afford **4l** as a pale yellow solid, 92% (418 mg) yield, mp 156 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 12.81 min, purity of 99%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 9.83 (s, 1H), 8.60 (dd, J = 7.5, 2.2 Hz, 1H), 8.04 (ddd, J = 8.8, 4.5, 2.3 Hz, 1H), 7.52 (dd, J = 11.3, 8.8 Hz, 1H), 6.46 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ 166.38, 155.06, 144.34, 137.37, 137.28, 133.25, 126.26, 126.23, 119.21, 119.00, 106.79; HRMS (ESI) for $C_9H_6FNO_5$ [M−H]$^-$: m/z calcd; 226.01570, found; 226.01590.

4.4.13. 2-Hydroxy-3-(4-methoxy-2-nitrophenyl)acrylic acid, ***4m***

Using general procedure-3, employing **2g** to afford **4m** as a yellow solid, 85% (406 mg) yield, mp 241 °C (dec.); RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 12.05 min, purity of 88%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 9.59 (bs, 1H), 8.34 (d, J = 2.3 Hz, 1H), 7.94 (dd, J = 8.9, 2.4 Hz, 1H), 7.34 (d, J = 8.9 Hz, 1H), 6.40 (s, 1H), 3.91 (s, 3H); HRMS (ESI) for $C_{10}H_9NO_6$ [M−H]$^-$: m/z calcd; 238.03570, found; 238.03590.

4.5. General procedure-4: synthesis of phenyl substituted α-bromoacetophenones, ***7***

1 mmol of the required acetophenone was dissolved in 10 mL of AcOH. Then 1 equiv. of bromine was dissolved in 10 mL of AcOH and then added drop wise to the reaction mixture. Then the reaction was stirred at room temperature for 4 h. The solvent was evaporated to dryness and the product was purified using normal phase

column chromatography with gradient increase of methanol in dichloromethane as eluent.

4.5.1. 4-(2-Bromoacetyl)benzoic acid, **7a**

Using general procedure-4, employing 4-acetylbenzoic acid to afford **7a** as a white solid, 80% (194 mg) yield, mp 217 °C; RP-HPLC (C18): 0–70% (ACN/DDW/0.1% TFA) in 25 min, t_R = 13.94 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.10 (d, J = 8.0 Hz, 2H), 8.01 (d, J = 8.0 Hz, 2H), 4.80 (s, 2H); ^{13}C NMR (100 MHz, DMSO) δ 191.92, 167.27, 138.45, 131.31, 130.27, 130.19, 129.91, 128.46, 66.31; HRMS (ESI) for $C_9H_7BrO_3$ $[M-H]^-$: m/z calcd; 240.95058, found; 240.95061.

4.5.2. 2-Bromo-1-(4-cyclohexylphenyl)ethanone, **7b**

Using general procedure-4, employing 1-(4-cyclohexylphenyl) ethan-1-one to afford **7b** as a brown oil, 50% (141 mg) yield; RP-HPLC (C18): 50–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.65 min, purity of 97%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.91 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 8.2 Hz, 2H), 2.54 (p, J = 16 Hz, 1H), 4.84 (s, 2H), 1.66–1.77 (m, 5H), 1.52–1.06 (m, 5H); ^{13}C NMR (DMSO, 100 MHz): δ 191.85, 145.63, 132.54, 129.68, 127.83, 44.53, 34.63, 34.16, 26.87, 26.16; LCMS (ESI) for $C_{14}H_{17}BrO$ $[M+H]^+$: m/z calcd; 281.05, found; 281.15.

4.6. 2-(2-Carbamothioylhydrazono)-3-(2-nitrophenyl)propanoic acid, **8**

5 mmol of 2-nitrophenyl pyruvic acid were dissolved in 10 mL of 5% AcOH/water, then 5 mmol of thiosemicarbazide were added and the reaction was allowed to reflux for 2 h. Then it was cooled to room temperature. A light yellow precipitate was formed, which was filtrated, washed with cold water and dried under vacuum: Yellow solid, mixture of two isomers (1:2/E:Z ratio), 98% (1.38 g) yield, mp 175 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 10.99, 11.95 min, purity of 100%; ^{1}H NMR (DMSO-*d6*, 500 MHz): *E-Isomer*: δ 11.21 (s, 1H), 8.82 (s, 2H), 8.05 (d, J = 7.5 Hz, 1H), 7.63 (t, J = 7.5 Hz, 1H), 7.51 (m, 2H), 6.98 (s, 1H), 4.36 (s, 2H); *Z-Isomer*: δ 12.13 (s, 1H), 8.76 (s, 2H), 7.96 (d, J = 7.5 Hz, 1H), 7.63 (m, 2H), 7.51 (t, J = 7.5 Hz, 1H), 6.96 (s, 1H), 4.09 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): *E-Isomer*: δ 180.70, 165.31, 149.51, 137.65, 134.50, 131.71, 129.69, 128.54, 125.77, 29.38; *Z-Isomer*: δ 179.28, 164.15, 150.05, 135.18, 134.02, 132.72, 132.18, 128.86, 125.08, 36.08; LCMS (ESI) for $C_{10}H_{10}N_4O_4S$ $[M+H]^+$: m/z calcd; 283.04, found; 282.96.

4.7. 4-(3,4-Dichloro-phenyl)-thiazol-2-yl-hydrazine, **9**

A solution of a 1 mmol of thiosemicarbazide and 1 mmol of 3,4-dichlorophenacyl bromide in dioxane (2 mL) was stirred at room temperature for 8 h. The precipitate of hydrobromide salt was filtered, washed with 1,4-dioxane (3 × 10 mL) then dissolved saturated $Na_2CO_{3(aq)}$ (10 mL) and stirred vigorously, the produced precipitate was filtered, washed with water and dried under vacuum. Yellow solid, 98% (0.253 g) yield, mp 107 °C; RP-HPLC (C18): 0–100% (ACN/DDW/0.1% TFA) in 25 min, t_R = 11.85 min, purity of 97%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 10.69 (s, 1H), 8.06 (d, J = 2.0 Hz, 1H), 7.80 (dd, J = 8.4, 2.1 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.43 (s, 1H), 4.89 (bs, 1H); ^{13}C NMR (100 MHz, DMSO) δ 170.78, 150.84, 136.16, 132.05, 131.49, 130.14, 127.82, 126.16, 106.17; HRMS (ESI) for $C_9H_7Cl_2N_3S$ $[M+H]^+$: m/z calcd; 259.98105, found; 259.98116.

4.8. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **4EGI-1**

1 mmol of the 2-(2-carbamothioylhydrazono)-3-(2-nitrophenyl) propanoic acid, **8**, and 1 mmol of 3,4-dichlorophenacyl bromide were dissolved in 2 mL of dry 1,4-dioxane, then allowed to react at room temperature for 8 h, a precipitate was formed, the precipitate was collected, washed with 1,4-dioxane and cold water then dried under vacuum. **4EGI-1** was obtained as mixture of E/Z isomers. The isomers were separated using reversed phase column chromatography using gradual increase of methanol in water containing 0.1% of formic acid as eluent. *E-Isomer*: Light pink solid, 30% (135 mg) yield, mp 202 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.68 min, purity of 100%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.50 (bs, 1H), 8.08 (d, J = 8.1 Hz, 1H), 8.04 (s, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.70–7.55 (m, 3H), 7.51 (t, J = 7.8 Hz, 1H), 7.10 (d, J = 7.8 Hz, 1H), 4.34 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 169.27, 166.12, 149.70, 148.58, 138.32, 135.47, 134.55, 132.20, 131.81, 131.53, 130.62, 129.59, 128.51, 127.90, 126.17, 125.76, 108.75, 29.97; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 451.00290, found; 451.00420. *Z-Isomer*: Creamy solid, 60% (270 mg) yield, mp 208 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1% TFA) in 25 min, t_R = 13.69 min, purity of 100%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.64 (s, 1H), 8.04 (dd, J = 8.1, 1.3 Hz, 1H), 8.00 (d, J = 2.0 Hz, 1H), 7.76 (dd, J = 8.5, 2.0 Hz, 1H), 7.69 (td, J = 7.5, 1.3 Hz, 1H), 7.60 (d, J = 8.4 Hz, 1H), 7.57–7.48 (m, 3H), 4.16 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 167.99, 164.75, 149.75, 148.40, 135.23, 134.20, 133.60, 132.60, 132.17, 131.41, 130.67, 128.86, 127.82, 126.23, 125.26, 108.46, 36.90; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 451.00290, found; 451.00440.

4.9. General procedure-5: synthesis of ring A modified thiazol-2-yl hydrazones, **10** and **11**

1 mmol of the required pyruvic acid, **5**, or 2-hydroxyacrylic acid, **4**, was dissolved in 2 mL of 5% acetic acid/ethanol, then 1 equiv. of 4-(3,4-dichloro-phenyl)-thiazol-2-yl-hydrazine, **9** (1.0 mmol, 259 mg) was added. The reaction mixture was heated to 90 °C and stirred for 1 h. Then it was cooled to 0 °C. The produced precipitate was filtered and washed with cold water then dried under vacuum. The products were obtained as mixture of E/Z isomers, which were separated using reversed phase column chromatography using gradual increase of methanol in water containing 0.1% of formic acid as eluent.

4.9.1. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(3-nitrophenyl)propanoic acid, **10a**

Using general procedure-5, employing **4a** to afford **10a**. *E-Isomer*: Yellow solid, 26% (117 mg) yield, mp 168 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.32 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.27 (bs, 1H), 8.13 (s, 1H), 8.08 (d, J = 2.0 Hz, 1H), 7.81 (td, J_1 = 10.2 Hz, J_2 = 2.0 Hz, 1H), 7.73–7.53 (m, 5H), 4.18 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 166.11, 163.58, 148.50, 139.21, 136.63, 135.82, 132.20, 131.62, 130.72, 130.31, 127.93, 126.25, 124.29, 123.74, 122.21, 121.62, 108.85, 31.46; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 451.00290, found; 451.00350. *Z-Isomer*: Yellow solid, 54% (243 mg) yield, mp 178 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 12.00 min, purity of 97%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 13.44 (bs, 1H), 8.13 (s, 1H), 8.10 (dd, J = 10.0 Hz, 1H), 8.05 (d, J = 1.9 Hz, 1H), 7.62 (d, J = 7.6 Hz, 1H), 7.61 (t, J = 10.0 Hz, 1H), 7.59 (s, 1H), 3.92 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.41, 163.68, 148.57, 148.33, 141.11, 138.66, 136.64, 135.47, 132.15, 131.46, 130.57, 130.28, 127.86, 126.31, 124.38, 121.93, 107.90, 39.13; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 451.00290, found; 451.00410.

4.9.2. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-nitrophenyl)propanoic acid, **10b**

Using general procedure-5, employing **4b** to afford **10b**. *E-Isomer*: Yellow solid, 20% (90 mg) yield, mp 196 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.16 min, purity of

95%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.50 (s, 2H), 8.19 (d, J = 7.9 Hz, 2H), 8.08 (s, 1H), 7.83 (d, J = 8.3 Hz, 1H), 7.66 (s, 1H), 7.49 (d, J = 8.3 Hz, 2H), 7.47 (d, J = 10.0 Hz, 1H), 4.23 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 166.04, 162.17, 146.85, 145.28, 132.21, 131.61, 130.84, 130.66, 130.06, 127.92, 126.31, 124.49, 124.27, 123.71, 108.46, 32.03; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 451.0029, found; 451.00555. *Z-Isomer*: Yellow solid, 63% (283 mg) yield, mp 211 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.86 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 13.56 (bs, 2H), 8.15 (d, J = 8.1 Hz, 2H), 8.04 (s, 1H), 7.8 (d, J = 8.3 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H), 7.56 (s, 1H), 7.51 (d, J = 8.1 Hz, 2H), 3.88 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.32, 163.74, 148.47, 147.22, 146.78, 138.33, 135.45, 132.17, 131.50, 130.88, 130.73, 127.91, 126.35, 123.99, 107.98, 39.57; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 451.00290, found; 451.00347.

4.9.3. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-phenylpropanoic acid, ***10c***

Using general procedure-5, employing phenylpyruvic acid to afford **10c**. *E-Isomer*: Yellow solid, 50% (202 mg) yield, mp 145 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.71 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 12.12 (bs, 2H), 8.09 (d, J = 2.0 Hz, 1H), 7.83 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H), 7.64–7.67 (m, 2H), 7.27–7.31 (m, 2H), 7.17–7.20 (m, 3H), 4.04 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 166.18, 163.23, 136.77, 132.17, 131.62, 129.23, 128.93, 127.91, 127.03, 126.24, 108.80, 31.69; HRMS (ESI) for $C_{18}H_{13}Cl_2N_3O_2S$ $[M+H]^+$: m/z calcd; 406.01840, found; 406.01780. *Z-Isomer*: Yellow solid, 25% (101 mg) yield, mp 197 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 13.80 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 13.49 (bs, 1H), 10.76 (bs, 1H), 8.08 (d, J = 2.1 Hz, 1H), 7.84 (q, J = 9.6 Hz, 2H), 7.66–7.62 (m, 2H), 7.44 (d, J_1 = 8.4 Hz, 1H), 7.20–7.33 (m, 3H), 3.76 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 165.69, 162.55, 138.90, 131.47, 29.48, 128.93, 127.83, 126.87, 126.16, 110.90, 47.78; HRMS (ESI) for $C_{18}H_{13}Cl_2N_3O_2S$ $[M+H]^+$: m/z calcd; 406.01840, found; 406.01783.

4.9.4. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-hydroxyphenyl)propanoic acid, ***10d***

Using general procedure-5, employing *p*-hydroxyphenylpyruvic acid to afford **10d**. *E-Isomer*: Yellow solid, 50% (210 mg) yield, mp 185 °C; RP-HPLC (C18): 0–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 16.63 min, purity of 99%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.11 (bs, 2H), 9.24 (s, 1H), 8.10 (s, 1H), 7.84 (d, J = 8.3 Hz, 1H), 7.66 (d, J = 8.4, 1H), 7.63 (s, 1H), 7.03 (d, J = 8.0 Hz, 2H), 6.70 (d, J = 8.0 Hz, 2H), 3.94 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 166.24, 163.09, 156.59, 141.60, 135.71, 132.20, 131.60, 130.01, 127.95, 126.65, 126.25, 116.01, 108.58, 30.78; HRMS (ESI) for $C_{18}H_{13}Cl_2N_3O_3S$ $[M+H]^+$: m/z calcd; 422.01270, found; 422.01410. *Z-Isomer*: Yellow solid, 40% (168 mg) yield, mp 183 °C; RP-HPLC (C18): 0–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 18.738 min, purity of 99%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 13.16 (bs, 1H), 9.20 (s, 1H), 8.07 (s, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.57 (s, 1H), 7.04 (d, J = 8.5 Hz, 2H), 6.68 (d, J = 8.2 Hz, 2H), 3.62 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.68, 165.95, 156.39, 148.60, 135.67, 132.15, 131.48, 130.49, 130.43, 128.98, 127.88, 126.35, 115.70, 110.91, 38.96; HRMS (ESI) for $C_{18}H_{13}Cl_2N_3O_3S$ $[M+H]^+$: m/z calcd; 422.01270, found; 422.01380.

4.9.5. 4-(2-Carboxy-2-(2-(5-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)ethyl)benzoic acid, ***10e***

Using general procedure-5, employing **4c** to afford **10e**. *E-Isomer*: Orange solid, 30% (134 mg) yield, mp 168 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 5.62, purity of 96%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.27 (bs, 3H), 8.08 (s, 1H), 7.89 (d, J = 8.0 Hz, 2H), 7.83 (d, J = 8.6 Hz, 1H), 7.64 (d, J = 8.8 Hz, 1H), 7.63 (s, 1H), 7.32 (d, J = 7.9 Hz, 2H), 4.14 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 169.46, 168.84, 166.11, 142.19, 140.07, 135.53, 132.21, 131.60, 130.60, 130.30, 129.65, 129.11, 127.92, 126.24, 108.71, 31.94; HRMS (ESI) for $C_{19}H_{13}Cl_2N_3O_4S$ $[M+H]^+$: m/z calcd; 450.00770, found; 450.00870. *Z-Isomer*: Orange solid, 40% (180 mg) yield, mp 185 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.13 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 12.86 (bs, 2H), 8.05 (s, 1H), 7.87 (d, J = 7.7 Hz, 2H), 7.81 (d, J = 8.5 Hz, 1H), 7.64 (s, 1H), 7.61 (d, J = 8.2 Hz, 1H), 7.34 (d, J = 7.8 Hz, 2H), 3.82 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 169.26, 167.88, 165.00, 143.56, 132.20, 131.53, 130.28, 130.09, 129.68, 129.10, 127.90, 127.83, 126.35, 126.16, 108.65, 36.51; HRMS (ESI) for $C_{19}H_{13}Cl_2N_3O_4S$ $[M+H]^+$: m/z calcd; 450.00770, found; 450.00910.

4.9.6. 3-(4-Aminophenyl)-2-(2-(4-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)propanoic acid, ***10f***

Using general procedure-5, employing **4d** to afford **10f**. *E-Isomer*: Yellow solid, 40% (96 mg) yield, mp 202 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 7.23 min, purity of 95%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.28 (s, 2H), 8.05 (d, J = 2.0 Hz, 1H), 7.83 (dd, J = 8.6, 1.9 Hz, 1H), 7.61 (dd, J = 8.4, 1.9 Hz, 1H), 7.43 (s, 1H), 6.90 (d, J = 8.0 Hz, 2H), 6.45 (d, J = 8.0 Hz, 2H), 3.48 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 165.72, 162.10, 149.90, 148.59, 147.41, 137.04, 136.01, 132.25, 131.41, 130.03, 127.79, 127.22, 127.10, 126.32, 114.41, 108.27, 35.02; HRMS (ESI) for $C_{18}H_{14}Cl_2N_4O_2S$ $[M+H]^+$: m/z calcd; 421.03010, found; 421.02800. *Z-Isomer*: Yellow solid, 20% (48 mg) yield, mp 212 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.74 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 13.63 (bs, 1H), 8.28 (s, 2H), 8.08 (d, J = 2.0 Hz, 1H), 7.84 (dd, J = 8.5, 2.0 Hz, 1H), 7.64 (dd, J= 8.4, 1.7 Hz, 1H), 7.59 (s, 1H), 6.93 (d, J = 8.2 Hz, 2H), 6.54 (d, J = 8.0 Hz, 2H), 3.56 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 165.68, 159.78, 148.70, 135.66, 132.16, 131.50, 130.51, 130.02, 129.47, 127.89, 126.65, 126.49, 126.37, 115.19, 107.52, 38.97; HRMS (ESI) for $C_{18}H_{14}Cl_2N_4O_2S$ $[M+H]^+$: m/z calcd; 421.03010, found; 421.02870.

4.9.7. 3-(4-Chlorophenyl)-2-(2-(4-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)propanoic acid, ***10g***

Using general procedure-5, employing **4e** to afford **10g**. *E-Isomer*: Yellow solid, 20% (88 mg) yield, mp 69 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 10.09 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.05 (d, J = 2.0 Hz, 1H), 7.82 (dd, J = 8.4, 2.1 Hz, 1H), 7.59 (d, J = 8.4 Hz, 1H), 7.47 (s, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.24 (d, J = 8.4 Hz, 2H), 4.04 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 163.2, 159.76, 134.11, 133.01, 131.91, 131.73, 131.46, 131.14, 131.14, 130.60, 129.73, 128.87, 128.55, 126.94, 124.37, 109.99, 34.55; HRMS (ESI) for $C_{18}H_{12}Cl_3N_3O_2S$ $[M+H]^+$: m/z calcd; 439.97886, found; 439.97929. *Z-Isomer*: Yellow solid, 60% (176 mg) yield, mp 102 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 14.81 min, purity of 97%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.07 (d, J = 2.0 Hz, 1H), 7.82 (dd, J = 8.4, 2.1 Hz, 1H), 7.63 (s, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.24 (d, J = 8.4 Hz, 2H), 3.73 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 165.26, 161.94, 142.38, 141.09, 137.64, 135.43, 132.17, 131.60, 131.53, 131.40, 130.61, 128.87, 127.88, 126.36, 119.78, 108.18, 38.93; HRMS (ESI) $C_{18}H_{12}Cl_3N_3O_2S$ $[M+H]^+$: m/z calcd; 439.97886, found; 439.97961.

4.9.8. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-fluorophenyl)propanoic acid, ***10h***

Using general procedure-5, employing **4f** to afford **10h**. *E-Isomer*: Yellow solid, 15% (63 mg) yield, mp 140 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 7.47 min, purity of 96%; HRMS (ESI) for $C_{18}H_{12}Cl_2FN_3O_2S$ $[M+H]^+$: m/z calcd; 422.01270, found; 422.01410. *Z-Isomer*: Yellow solid, 70% (295 mg)

yield, mp 182 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 13.16 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.06 (d, J = 2.0 Hz, 1H), 7.84 (dd, J = 8.5, 2.1 Hz, 1H), 7.63 (d, J = 8.6 Hz, 1H), 7.46 (s, 1H), 7.29 (dd, J = 8.4, 5.6 Hz, 2H), 7.06 (t, J = 8.8 Hz, 2H), 3.68 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 169.29, 166.61, 146.52, 136.05, 132.07, 131.30, 130.20, 127.79, 126.31, 115.34, 115.10, 110.93, 110.91, 106.07, 39.34; HRMS (ESI) for $C_{18}H_{12}Cl_2FN_3O_2S$ $[M+H]^+$: m/z calcd; 422.01270, found; 422.01380.

4.9.9. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-(trifluoromethyl)phenyl)propanoic acid, **10i**

Using general procedure-5, employing **4g** to afford **10i**. *E-Isomer*: White solid, 45% (213 mg) yield, mp 166 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 10.60 min, purity of 97%; ^{1}H NMR (Acetone-d_6, 500 MHz): δ 8.04 (d, J = 2.0 Hz, 1H), 7.83 (dd, J = 8.4, 2.1 Hz, 1H), 7.69 (d, J = 8.0 Hz, 2H), 7.60 (s, 1H), 7.59 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 1H), 4.36 (s, 2H); ^{13}C NMR (Acetone-d_6, 125 MHz): 168.20, 164.91, 148.99, 143.06, 138.94, 135.37, 132.34, 130.95, 130.91, 130.03, 129.36, 127.78, 125.77, 125.67, 125.33, 107.29, 30.93; ^{19}F NMR (Acetone-d_6, 470 MHz) δ −76.47; HRMS (ESI) for $C_{19}H_{12}Cl_2F_3N_3O_2S$ $[M+H]^+$: m/z calcd; 474.00521, found; 474.00560. *Z-Isomer*: White solid, 53% (251 mg) yield, mp 185 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1% TFA) in 25 min, t_R = 15.12 min, purity of 98%; ^{1}H NMR (Acetone-d$_6$, 500 MHz): δ 8.09 (d, J = 2.0 Hz, 1H), 7.88 (dd, $J_=$ 8.4, 2.1 Hz, 1H), 7.70 (d, J = 8.0 Hz, 2H), 7.60 (d, J = 8.4 Hz, 2H), 7.60 (s, 1H), 7.53 (s, 1H), 4.01 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.03, 164.99, 143.28, 135.28, 132.20, 131.52, 130.71, 130.34, 129.72, 127.90, 127.66, 126.34, 125.84, 125.80, 125.76, 108.60, 39.30; ^{19}F NMR (Acetone-d_6, 470 MHz) δ −63.21; HRMS (ESI) for $C_{19}H_{12}Cl_2F_3N_3O_2S$ $[M+H]^+$: m/z calcd; 474.00521, found; 474.00577.

4.9.10. 3-(4-Amino-3-nitrophenyl)-2-(2-(5-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)propanoic acid, **11a**

Using general procedure-5, employing **4h** to afford **11a**. *E-Isomer*: Yellow solid, 20% (93 mg) yield, mp 170 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 12.30 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.11 (bs, 1H), 8.09 (s, 1H), 7.84 (d, J = 10.0 Hz, 1H), 7.83 (s, 1H), 7.64 (d, J = 10.0 Hz, 1H), 7.63 (s, 1H), 7.36 (bs, 2H), 7.28 (d, J = 10.0 Hz, 1H), 6.96 (d, J = 10.0 Hz, 1H), 3.90 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 169.50, 166.25, 148.71, 145.76, 137.27, 135.54, 132.21, 131.61, 130.61, 130.54, 127.94, 126.27, 124.98, 123.77, 120.26, 108.77, 30.27; HRMS (ESI) for $C_{18}H_{13}Cl_2N_5O_4S$ $[M+H]^+$: m/z calcd; 466.01381, found; 466.01470. *Z-Isomer*: Yellow solid, 60% (280 mg) yield, mp 199 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 15.09 min, purity of 99%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.33 (s, 2H), 8.08 (s, 1H), 7.83 (s, 1H), 7.68–7.61 (m, 2H), 7.44 (s, 1H), 7.36 (s, 2H), 7.31 (d, J = 8.7 Hz, 1H), 6.98 (d, J = 8.6 Hz, 1H), 3.66 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 168.39, 165.31, 148.57, 145.68, 137.80, 135.48, 132.18, 131.53, 130.57, 127.89, 126.36, 125.66, 125.43, 119.88, 108.06, 38.24; HRMS (ESI) for $C_{18}H_{13}Cl_2N_5O_4S$ $[M+H]^+$: m/z calcd; 466.01381, found; 466.01253.

4.9.11. 4-(2-Carboxy-2-(2-(5-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)ethyl)-3-nitrobenzoic acid, **11b**

Using general procedure-5, employing **4i** to afford **11b**. *E-Isomer*: Yellow solid, 22% (109 mg) yield, mp 164 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.81 min, purity of 96%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.49 (s, 1H), 8.15 (d, J = 8.0 Hz, 1H), 8.02 (s, 1H), 7.77 (d, J = 8.3 Hz, 1H), 7.65 (s, 1H), 7.61 (d, J = 8.4 Hz, 1H), 7.21 (d, J = 8.1 Hz, 1H), 4.34 (s, 2H); ^{13}C NMR (100 MHz, DMSO) δ 169.20, 166.09, 166.03, 149.61, 137.66, 136.63, 135.44, 134.74, 132.17, 131.59, 131.22, 130.60, 130.31, 127.88, 126.27, 126.19, 108.88, 30.32; HRMS (ESI) for $C_{19}H_{12}Cl_2N_4O_6S$ $[M+Na]^+$: m/z calcd; 516.97470, found 516.97550. *Z-Isomer*: Yellow solid, 58% (397 mg) yield, mp 192 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 12.48 min, purity of 98%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.49 (s, 1H), 8.19 (d, J = 9.0 Hz, 1H), 7.98 (s, 1H), 7.74 (d, J = 8.4 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.50 (s, 1H), 4.24 (s, 2H); ^{13}C NMR (100 MHz, DMSO) δ 168.02, 166.22, 164.73, 149.68, 148.36, 137.53, 135.23, 134.34, 134.26, 132.15, 131.47, 130.64, 127.81, 126.25, 125.82, 108.45, 37.07; HRMS (ESI) for $C_{19}H_{12}Cl_2N_4O_6S$ $[M+Na]^+$: m/z calcd; 516.97470, found; 516.97550.

4.9.12. 2-(2-(5-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-hydroxy-3-nitrophenyl)propanoic acid, **11c**

Using general procedure-5, employing **4j** to afford **11c**. *E-Isomer*: Yellow solid, 37% (172 mg) yield, mp 141 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 7.82 min, purity of 97%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.84 (s, 1H), 8.11 (s, 1H), 7.85 (dd, J = 8.6, 2.3 Hz, 1H), 7.82 (d, J = 2.2 Hz, 1H), 7.66 (d, J = 7.9 Hz, 1H), 7.57 (s, 1H), 7.42 (dd, J = 8.7, 2.2 Hz, 1H), 7.10 (d, J = 8.5 Hz, 1H), 4.01 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 168.69, 166.16, 151.33, 136.91, 136.84, 136.28, 132.12, 131.49, 130.44, 127.84, 126.34, 125.70, 125.30, 120.11, 107.39, 34.38; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_5S$ $[M-H]^-$: m/z calcd; 464.98330, found; 464.98260. *Z-Isomer*: Yellow solid, 60% (280 mg) yield, mp 190 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.29 min, purity of 99%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.95 (s, 1H), 10.84 (s, 1H), 8.06 (s, 1H), 7.82 (dd, J = 8.6, 2.3 Hz, 1H), 7.77 (d, J = 2.2 Hz, 1H), 7.64 (s, 1H), 7.63 (d, J = 7.9, 1H), 7.43 (dd, J = 8.7, 2.2 Hz, 1H) 7.10 (d, J = 8.5 Hz, 1H), 3.76 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.19, 165.10, 151.53, 148.56, 136.94, 136.87, 135.35, 132.20, 131.51, 130.68, 129.44, 127.90, 126.34, 125.83, 119.76, 108.43, 38.09; HRMS (ESI) for $C_{18}H_{12}Cl_2N_4O_5S$ $[M-H]^-$: m/z calcd; 464.98330, found; 464.98310.

4.9.13. 2-(2-(5-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-methoxy-2-nitrophenyl)propanoic acid, **11d**

Using general procedure-5, employing **4m** to afford **11d**. *E-Isomer*: Yellow solid, 25% (120 mg) yield, mp 92 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1% TFA) in 25 min, t_R = 8.01 min, purity of 95%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.05 (s, 1H), 7.80 (d, J = 6.5 Hz, 1H), 7.73 (s, 1H), 7.62 (d, J = 8.3 Hz, 1H), 7.55 (s, 1H), 7.28 (d, J = 8.4 Hz, 1H), 7.28 (d, J = 8.4 Hz, 1H), 3.87 (s, 3H), 3.86 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 170.78, 168.65, 165.53, 150.88, 148.43, 139.49, 136.05, 135.74, 132.04, 131.48, 127.81, 126.16, 125.84, 114.77, 106.15, 57.30, 25.53; HRMS (ESI) for $C_{19}H_{14}Cl_2N_4O_5S$ $[M-H]^-$: m/z calcd; 478.99890, found; 479.00060. *Z-Isomer*: Yellow solid, 60% (288 mg) yield, mp 105 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 10.68 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.07 (s, 1H), 7.82 (d, J = 6.5 Hz, 1H), 7.76 (s, 1H), 7.65 (d, J = 8.3 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.43 (s, 1H), 7.31 (d, J = 8.4 Hz, 1H), 3.91 (s, 3H), 3.80 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 170.74, 168.65, 166.13, 151.21, 148.43, 139.49, 136.14, 135.08, 132.04, 130.46, 130.14, 126.33, 125.84, 125.43, 115.27, 107.44, 57.30, 38.35; HRMS (ESI) for $C_{19}H_{14}Cl_2N_4O_5S$ $[M+H]^+$: m/z calcd; 481.01350, found; 481.01440.

4.9.14. 3-(4-Bromo-2-nitrophenyl)-2-(2-(4-(3,4-dichlorophenyl)thiazol-2-yl)hydrazono)propanoic acid, **11e**

Using general procedure-5, employing 3-(4-bromo-2-nitrophenyl)-2-oxopropanoic acid [16] to afford **11e**. *E-Isomer*: Yellow solid, 10% (53 mg) yield, mp 166 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 15.54 min, purity of 95%; HRMS (ESI) for $C_{18}H_{11}BrCl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 530.91110, found; 530.91230. *Z-Isomer*: Yellow solid, 80% (425 mg) yield, mp 168 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 18.04 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.22

(d, J = 2.1 Hz, 1H), 7.99 (d, J = 2.0 Hz, 1H), 7.89 (dd, J = 8.2, 2.1 Hz, 1H), 7.75 (dd, J = 8.4, 2.1 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.52 (s, 1H), 7.47 (d, J = 8.3 Hz, 1H), 4.12 (s, 2H); ^{13}C NMR (100 MHz, DMSO) δ 168.05, 164.67, 150.43, 148.42, 136.78, 135.46, 135.27, 134.95, 132.18, 132.03, 131.46, 130.68, 127.85, 127.67, 126.28, 120.57, 108.45, 36.38; HRMS (ESI) for $C_{18}H_{11}BrCl_2N_4O_4S$ $[M+H]^+$: m/z calcd; 530.91110, found; 530.91270.

4.9.15. 3-(4-Chloro-3-nitrophenyl)-2-(2-(4-(3,4-dichlorophenyl) thiazol-2-yl)hydrazono)propanoic acid, **11f**

Using general procedure-5, employing **4k** to afford **11f**. *E-Isomer*: Yellow solid, 30% (144 mg) yield, mp 188 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 9.44 min, purity of 100%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.03 (d, J = 2.0 Hz, 1H), 7.94 (d, J = 2.2 Hz, 1H), 7.79 (dd, J = 8.4, 2.2 Hz, 1H), 7.69 (d, J = 8.3 Hz, 1H), 7.62 (s, 1H), 7.61 (d, J = 8.2 Hz, 1H), 7.56 (dd, J = 8.4, 2.2 Hz, 1H), 3.88 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 166.01, 164.79, 148.07, 146.54, 139.56, 135.39, 134.43, 132.38, 132.20, 131.97, 131.50, 130.73, 127.91, 126.75, 126.32, 123.50, 108.60, 38.26; HRMS (ESI) for $C_{18}H_{11}Cl_3N_4O_4S$ $[M+H]^+$: m/z calcd; 484.96394, found; 484.96320. *Z-Isomer*: Yellow solid, 45% (218 mg) yield, mp 208 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 13.13 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 500 MHz): 8.05 (s, 1H), 7.94 (s, 1H), 7.82 (d, J = 8.3 Hz, 1H), 7.69 (d, J = 8.2 Hz, 1H), 7.62 (d, J = 8.2 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.54 (s, 1H), 3.76 (d, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.72, 165.52, 148.58, 147.93, 140.76, 135.69, 135.35, 132.12, 131.83, 131.43, 130.43, 127.84, 127.76, 126.66, 126.25, 123.14, 107.26, 39.00; HRMS (ESI) for $C_{18}H_{11}Cl_3N_4O_4S$ $[M+H]^+$: m/z calcd; 484.96394, found; 484.96404.

4.9.16. 2-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazono)-3-(4-fluoro-3-nitrophenyl)propanoic acid, **11g**

Using general procedure-5, employing **4l** to afford **11g**. *E-Isomer*: Yellow solid, 34% (160 mg) yield, mp 140 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.40 min, purity of 95%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.10 (d, J = 1.4 Hz, 1H), 7.80 (dt, J = 7.0, 1.2 Hz, 2H), 7.68–7.60 (m, 2H), 7.60 (d, J = 3.2 Hz, 1H), 7.45–7.38 (m, 1H), 4.10 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 166.59, 163.97, 150.85, 148.32, 140.16, 137.75, 137.13, 132.05, 131.43, 128.50, 127.88, 127.79, 126.74, 126.14, 119.86, 118.78, 106.10, 25.50; HRMS (ESI) for $C_{18}H_{11}Cl_2FN_4O_4S$ $[M+H]^+$: m/z calcd; 468.99350, found; 468.99440. *Z-Isomer*: Yellow solid, 48% (225 mg) yield, mp 190 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.59 min, purity of 97%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.89 (bs, 1H), 8.07 (d, J = 1.4 Hz, 1H), 7.89–7.79 (m, 1H), 7.85–7.75 (m, 1H), 7.69 (s, 1H), 7.69 (d, J = 8.6 Hz, 1H), 7.65 (s, 1H), 7.57 (d, J = 8.6 Hz, 1H), 3.92 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.17, 164.93, 155.09, 148.57, 137.84, 137.22, 135.87, 135.32, 132.24, 131.53, 130.75, 127.92, 127.11, 126.35, 118.98, 118.82, 108.57, 38.12; HRMS (ESI) for $C_{18}H_{11}Cl_2FN_4O_4S$ $[M+H]^+$: m/z calcd; 468.99350, found; 468.99481.

4.10. General procedure-6: synthesis of ring C modified thiazol-2-yl hydrazones, **12**

1 mmol of 2-(2-carbamothioylhydrazono)-3-(2-nitrophenyl) propanoic acid, **8**, and 1 mmol of the required α-bromoacetophenone were dissolved in 2 mL of dry 1,4-dioxane, then allowed to react at room temperature for 8 h, a precipitate was formed. The precipitate was collected, washed with 1,4-dioxane and cold water then dried under vacuum. The products were obtained as mixture of E/Z isomers, which were separated using reverse phase column chromatography using gradual increase of methanol in water containing 0.1% of formic acid as eluent.

4.10.1. 3-(2-Nitrophenyl)-2-(2-(4-phenylthiazol-2-yl)hydrazono) propanoic acid, **12a**

Using general procedure-6, employing 2-bromo-1-phenylethan-1-one to afford **12a**. *E-Isomer*: Yellow solid, 40% (152 mg) yield, mp 155 °C; RP-HPLC (C18): 0–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 15.80 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 12.10 (bs, 2H), 8.06 (d, J = 8.3 Hz, 1H), 7.80 (d, J = 7.4 Hz, 2H), 7.64 (t, J = 7.6 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.43 (s, 1H), 7.37 (t, J = 7.6 Hz, 1H), 7.30 (d, J = 6.5 Hz, 1H), 7.29 (d, J = 7.3 Hz, 1H), 7.07 (d, J = 7.7 Hz, 1H), 4.31 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 165.75, 149.54, 134.41, 132.11, 131.86, 131.57, 129.59, 129.35, 128.41, 128.33, 126.19, 106.20, 30.19; HRMS (ESI) for $C_{18}H_{14}N_4O_4S$ $[M+H]^+$: m/z calcd; 383.08085, found; 383.08104. *Z-Isomer*: Yellow solid, 56% (214 mg) yield, mp 180 °C; RP-HPLC (C18): 0–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 16.98 min, purity of 99%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 12.83 (bs, 1H), 8.05 (d, J = 7.9 Hz, 1H), 7.78 (d, J = 7.9 Hz, 2H), 7.70 (t, J = 7.5 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.52 (d, J = 8.0 Hz, 1H), 7.37 (t, J = 8.0 Hz, 2H), 7.33 (s, 1H), 7.28 (t, J = 8.0 Hz, 1H), 4.18 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 164.76, 149.79, 134.24, 133.62, 132.70, 129.31, 128.87, 128.52, 126.24, 125.27, 106.21, 36.85; HRMS (ESI) for $C_{18}H_{14}N_4O_4S$ $[M+H]^+$: m/z calcd; 383.08085, found; 383.08163.

4.10.2. 2-(2-(4-(4-Hydroxyphenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12b**

Using general procedure-6, employing 2-bromo-1-(4-hydroxyphenyl)ethan-1-one to afford **12b**. *E-Isomer*: Yellow solid, 40% (160 mg) yield, mp 125 °C; RP-HPLC (C18): 0–70% (ACN/Water/0.1%TFA) in 25 min, t_R = 16.48 min, purity of 98%; HRMS (ESI) for $C_{18}H_{14}N_4O_5S$ $[M+H]^+$: m/z calcd; 399.07577, found; 399.07691. *Z-Isomer*: Yellow solid, 60% (238 mg) yield, mp 196 °C; RP-HPLC (C18): 0–70% (ACN/Water/0.1%TFA) in 25 min, t_R = 17.90 min, purity of 96%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.85 (bs, 1H), 9.58 (s, 1H), 7.72 (t, J = 7.5 Hz, 1H), 7.60 (d, J = 8.5 Hz, 2H), 7.56 (t, J = 8.0 Hz, 1H), 7.54 (d, J = 8.5 Hz, 1H), 7.06 (s, 1H), 6.77 (d, J = 8.6 Hz, 2H), 4.18 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 170.99, 164.77, 158.06, 149.84, 134.24, 133.62, 132.62, 128.86, 127.71, 125.27, 116.04, 36.82; HRMS (ESI) for $C_{18}H_{14}N_4O_5S$ $[M+H]^+$: m/z calcd; 399.07577, found; 399.07606.

4.10.3. 4-(2-(2-(1-Carboxy-2-(2-nitrophenyl)ethylidene) hydrazinyl)thiazol-4-yl)benzoic acid, **12c**

Using general procedure-6, employing 4-(2-bromoacetyl)benzoic acid to afford **12c**. *E-Isomer*: Yellow solid, 5% (21 mg) yield, mp 211 °C (dec.); RP-HPLC (C18): 0–70% (ACN/Water/0.1%TFA) in 25 min, t_R = 16.67 min, purity of 97%; HRMS (ESI) for $C_{19}H_{14}N_4O_6S$ $[M+H]^+$: m/z calcd; 427.07068, found; 427.07014. *Z-Isomer*: Yellow solid, 58% (247 mg) yield, mp 207 °C (dec.); RP-HPLC (C18): 0–70% (ACN/Water/0.1%TFA) in 25 min, t_R = 18.97 min, purity of 98%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.15 (s, 1H), 8.06 (d, J = 8.2 Hz, 1H), 7.98–7.88 (m, 4H), 7.71 (t, J = 7.5 Hz, 1H), 7.57 (d, J = 7.8 Hz, 1H), 7.54 (s, 1H), 7.53 (s, 2H), 4.19 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.25, 167.74, 165.01, 163.02, 149.90, 138.70, 134.20, 133.62, 132.93, 130.46, 130.31, 128.80, 126.25, 125.23, 108.47, 37.011; HRMS (ESI) for $C_{19}H_{14}N_4O_6S$ $[M+H]^+$: m/z calcd; 427.07068, found; 427.07090.

4.10.4. 2-(2-(4-(4-Aminophenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12d**

Using general procedure-6, employing 1-(4-aminophenyl)-2-bromoethan-1-one to afford **12d**. *E-Isomer*: Yellow solid, 18% (68 mg) yield, mp 124 °C (dec.); RP-HPLC (C18): 0–70% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.07 min, purity of 97%; HRMS (ESI) for $C_{18}H_{15}N_5O_4S$ $[M+H]^+$: m/z calcd; 398.09180, found; 398.09300. *Z-Isomer*: Yellow solid, 60% (238 mg) yield, mp 157 °C (dec.); RP-HPLC

(C18): 0–70% (ACN/Water/0.1%TFA) in 25 min, t_R = 15.23 min, purity of 98%; ^{1}H NMR (DMSO-d$_6$, 500 MHz): δ 7.99 (d, J = 8.1 Hz, 1H), 7.73 (d, J = 8.3 Hz, 1H), 7.65 (t, J = 8.3 Hz, 1H), 7.58 (d, J = 8.2 Hz, 1H), 7.43–7.52 (m, 5H), 6.55 (d, J = 8.3 Hz, 2H), 4.11 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 161.83, 160.23, 149.97, 148.73, 134.50, 133.95, 133.39, 128.45, 127.24, 126.77, 125.04, 119.99, 119.77, 114.57, 109.52, 36.68; HRMS (ESI) for $C_{18}H_{15}N_5O_4S$ $[M+H]^+$: m/z calcd; 398.09180, found; 398.09270.

4.10.5. 2-(2-(4-(4-Morpholinophenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12e**

Using general procedure-6, employing 2-bromo-1-(4-morpholinophenyl)ethan-1-one to afford **12e**. *E-Isomer*: Yellow solid, 20% (93 mg) yield; RP-HPLC (C18): 0–100% (ACN/Water/0.1% TFA) in 25 min, t_R = 15.08 min, purity of 100%; HRMS (ESI) for $C_{22}H_{21}N_5O_5S$ $[M+H]^+$: m/z calcd; 468.13362; found; 468.13440. *Z-Isomer*: Yellow solid, 70% (333 mg) yield, mp 177 °C; RP-HPLC (C18): 0–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 17.70 min, purity of 98%; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ 7.68 (t, J = 8.0 Hz, 2H), 7.63 (d, J = 8.0 Hz, 2H), 7.53 (t, J = 8.0 Hz, 1H), 7.51 (d, J = 7.0 Hz, 1H), 7.03 (s, 1H), 6.91 (d, J = 8.0 Hz, 2H), 4.16 (s, 2H), 3.69 (p, J = 7.9, 6.0 Hz, 4H), 3.08 (p, J = 7.8, 6.1 Hz, 4H); ^{13}C NMR (DMSO, 100 MHz): δ 168.27, 164.73, 151.25, 149.84, 134.12, 133.53, 132.83, 128.76, 127.11, 125.20, 115.34, 102.78, 66.70, 48.71, 36.80; HRMS (ESI) for $C_{22}H_{21}N_5O_5S$ $[M+H]^+$: m/z calcd; 468.13362, found; 468.13322.

4.10.6. 3-(2-Nitrophenyl)-2-(2-(4-(4-(pyrrolidin-1-yl)phenyl) thiazol-2-yl)hydrazono)propanoic acid, **12f**

Using general procedure-6, employing 2-bromo-1-(4-(pyrrolidin-1-yl)phenyl)ethan-1-one to afford **12f**. Was obtained as mixture of E/Z isomers (1:1 ratio) brown solid, 97% (437 mg) yield, mp 179 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.83, 10.29 min, purity of 100%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.06 (dd, J = 8.1, 3.1 Hz, 2H), 7.70 (t, J = 7.5 Hz, 1H), 7.65 (t, J = 7.6 Hz, 1H), 7.61 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 8.5 Hz, 2H), 7.52 (dt, J = 15.9, 8.5 Hz, 3H), 7.09 (d, J = 7.8 Hz, 1H), 7.00 (s, 1H), 6.91 (s, 1H), 6.52 (d, J = 8.4 Hz, 4H), 4.32 (s, 2H), 4.19 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 166.27, 164.59, 149.82, 149.77, 148.05, 147.91, 134.46, 134.17, 133.57, 132.79, 132.01, 129.67, 128.82, 128.43, 127.29, 127.25, 125.66, 125.24, 112.21, 100.83, 48.00, 36.74, 29.77, 25.63; HRMS (ESI) for $C_{22}H_{21}N_5O_4S$ $[M+H]^+$: m/z calcd; 452.13870, found; 452.14030.

4.10.7. 2-(2-(4-(4-Cyclohexylphenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12g**

Using general procedure-6, employing 2-bromo-1-(4-cyclohexylphenyl)ethan-1-one to afford **12g**. *E-Isomer*: Yellow solid, 15% (69 mg) yield, mp 122 °C (dec.); RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.64 min, purity of 98%; HRMS (ESI) for $C_{24}H_{24}N_4O_4S$ $[M+H]^+$: m/z calcd; 465.1591, found; 465.15980. *Z-Isomer*: Yellow solid, 63% (292 mg) yield, mp 168 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 13.86 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.04 (d, J = 8.2 Hz, 1H), 7.68 (t, J = 8.0 Hz, 1H), 7.67 (d, J = 8.0 Hz, 2H), 7.56 (t, J = 8.0 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.24 (s, 1H), 7.20 (d, J = 8.8 Hz, 2H), 4.17 (s, 2H), 2.47 (d, J = 9.1 Hz, 1H), 1.75 (d, J = 10.1 Hz, 4H), 1.67 (d, J = 14.2 Hz, 1H), 1.35 (h, J = 12.3, 11.9 Hz, 4H), 1.21 (t, J = 11.9 Hz, 1H); ^{13}C NMR (DMSO, 100 MHz): δ 164.72, 159.56, 149.79, 148.03, 134.22, 133.59, 132.70, 128.86, 127.56, 126.24, 125.26, 44.19, 36.81, 34.52, 27.01, 26.26; HRMS (ESI) for $C_{24}H_{24}N_4O_4S$ $[M+H]^+$: m/z calcd; 465.15910, found; 465.16026.

4.10.8. 2-(2-(4-([1,1′-Biphenyl]-4-yl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12h**

Using general procedure-6, employing 1-([1,1′-biphenyl]-4-yl)-2-bromoethan-1-one to afford **12h**. Yellow solid, precipitated from reaction mixture as the pure *Z-isomer*: 98% (448 mg) yield, mp 190 °C; RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 10.23 min, purity of 97%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 12.82 (s, 1H), 8.06 (d, J = 8.1 Hz, 1H), 7.88 (d, J = 8.2 Hz, 2H), 7.68 (d, J = 7.0 Hz, 4H), 7.59–7.50 (m, 2H), 7.44 (t, J = 7.6 Hz, 2H), 7.40 (s, 1H), 7.34 (t, J = 7.3 Hz, 1H), 4.26–4.10 (m, 2H); ^{13}C NMR (100 MHz, DMSO) δ 164.74, 149.79, 140.21, 140.06, 134.25, 133.63, 132.66, 129.63, 128.89, 128.21, 127.53, 127.18, 126.82, 125.29, 106.43, 36.85; HRMS (ESI) for $C_{24}H_{18}N_4O_4S$ $[M+H]^+$: m/z calcd; 459.11215, found; 459.11304.

4.10.9. 2-(2-(4-(Naphthalen-2-yl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12i**

Using general procedure-6, employing 2-bromo-1-(naphthalen-2-yl)ethan-1-one to afford **12i**. *E-Isomer*: Yellow solid, 30% (129 mg) yield, mp 174 °C (dec.); RP-HPLC (C18): 50–100% (ACN/Water/0.1% TFA) in 25 min, t_R = 7.27 min, purity of 95%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.32 (s, 1H), 8.07 (d, J = 8.1 Hz, 1H), 7.98–7.84 (m, 4H), 7.65 (t, J = 7.5 Hz, 1H), 7.58 (s, 1H), 7.54–7.44 (m, 4H), 4.32 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 166.18, 164.20, 149.71, 138.29, 134.56, 134.17, 132.17, 131.86, 129.62, 129.35, 129.23, 128.47, 126.19, 125.72, 124.43, 124.16, 106.55, 29.86; HRMS (ESI) for $C_{22}H_{16}N_4O_4S$ $[M+H]^+$: m/z calcd; 433.09650, found; 433.09590. *Z-Isomer*: Yellow solid, 60% (260 mg) yield, mp 190 °C (dec.); RP-HPLC (C18): 50–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 8.99 min, purity of 97%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.86 (bs, 1H), 8.37 (s, 1H), 8.08 (d, J = 8.1 Hz, 1H), 7.99–7.84 (m, 4H), 7.72 (t, J = 7.5 Hz, 1H), 7.60–7.41 (m, 5H), 4.22 (s, 2H); ^{13}C NMR (DMSO, 125 MHz): δ 168.13, 164.82, 149.84, 134.22, 133.84, 133.64, 133.22, 132.70, 128.88, 128.82, 128.24, 127.17, 126.82, 125.27, 124.97, 124.47, 106.94, 36.86; HRMS (ESI) for $C_{22}H_{16}N_4O_4S$ $[M+H]^+$: m/z calcd; 433.09650, found; 433.09700.

4.10.10. 2-(2-(4-(4-Azidophenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12j**

Using general procedure-6, employing 1-(4-azidophenyl)-2-bromoethan-1-one to afford **12j**. *E-Isomer*: Yellow solid, 20% (84 mg) yield, mp 160 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 11.72 min, purity of 96%; ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.05 (d, J = 8.2 Hz, 1H), 7.87–7.78 (m, 2H), 7.63 (t, J = 7.6 Hz, 1H), 7.49 (t, J = 7.8 Hz, 1H), 7.39 (d, J = 1.9 Hz, 1H), 7.16–7.02 (m, 3H), 4.30 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.09, 143.83, 139.15, 136.68, 134.32, 133.08, 132.44, 130.35, 124.72, 110.86, 34.67; HRMS (ESI) for $C_{18}H_{13}N_7O_4S$ $[M+H]^+$: m/z calcd; 424.08220, found; 424.08350. *Z-Isomer*: Yellow solid, 58% (250 mg) yield, mp 185 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 13.53 min, purity of 95%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.76 (s, 1H), 8.07 (dd, J = 8.2, 1.3 Hz, 1H), 7.87–7.82 (m, 2H), 7.72 (td, J = 7.5, 1.4 Hz, 1H), 7.60–7.51 (m, 2H), 7.36 (s, 1H), 7.17–7.10 (m, 2H), 4.20 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 164.71, 149.78, 139.34, 134.26, 133.64, 132.61, 128.91, 127.87, 125.28, 120.06, 106.17, 36.85; HRMS (ESI) for $C_{18}H_{13}N_7O_4S$ $[M+H]^+$: m/z calcd; 424.08220, found; 424.08350.

4.10.11. 2-(2-(4-(4-Bromophenyl)thiazol-2-yl)hydrazono)-3-(2-nitrophenyl)propanoic acid, **12k**

Using general procedure-6, employing 2-bromo-1-(4-bromophenyl)ethan-1-one to afford **12k**. *E-Isomer*: Yellow solid, 40% (185 mg) yield, mp 189 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 12.48 min, purity of 96%; HRMS (ESI) for $C_{18}H_{13}BrN_4O_4S$ $[M+H]^+$: m/z calcd; 462.9894, found; 462.9903. *Z-Isomer*: Yellow solid, 55% (254 mg) yield, mp 204 °C; RP-HPLC (C18): 30–100% (ACN/Water/0.1%TFA) in 25 min, t_R = 14.19 min, purity of 98%; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.04 (d, J = 8.1 Hz, 1H), 7.73 (d, J = 8.5 Hz, 2H), 7.69 (t, J = 7.6 Hz, 1H), 7.50–7.56 (m, 4H), 7.40 (s, 1H), 4.17 (s, 2H); ^{13}C NMR (DMSO, 100 MHz): δ 168.02, 164.76, 149.77, 144.33, 134.24, 133.84, 133.62,

132.62, 132.22, 129.29, 128.89, 128.26, 125.27, 121.52, 107.16, 36.87; HRMS (ESI) for $C_{18}H_{13}BrN_4O_4S$ $[M+H]^+$: *m/z* calcd; 462.9894, found; 462.9908.

5. Biology assays

5.1. Plasmid construction, expression, and purification

The fusion protein GST-eIF4E, which is markedly more soluble than the native eIF4E, was constructed by cloning the full coding sequence of murine eIF4E into a GST expression vector between the BamHI and EcoRI restriction sites. EIF4G-derived peptide binds to GST-eIF4E with the same binding affinity as it binds to the native eIF4E. The sequence of murine eIF4E is identical to human eIF4E in the structured portion of the protein with the exception of the substitution of an E for D174, which is a non-conserved residue. A GST-eIF4E construct of full length human eIF4E was transformed into *E. Coli* BL21(DE3) competent cells. The bacteria were grown at 37 °C in LFB media until $OD_{600} = 0.6$ and then the temperature was lowered to 20 °C and cells were induced with 0.1 mM IPTG overnight. The pellet from 2 L of culture was re-suspended in 40 ml of a buffer containing 50 mM Tris–HCl pH 7.5, 125 mM NaCl, 25 μg/mL lysozyme, 1% Triton-X, 10 mM β-mercaptoethanol, a protease inhibitor tablet, and lysed in a micro-fluidizer. The lysate was centrifuged at 40,000*g* for 1.5 h and supernatant was passed through a 0.22 μm pore filter and applied on an adipic dihydrazide agarose beads column coupled with m^7GDP which was prepared as described [27]. The column was washed five times with 25 mL of a buffer consisting of 10 mM HEPES (pH 7.5), 125 mM NaCl, 1 mM TCEP and eluted with 25 mL of the same buffer with an addition of 100 μM m^7GTP. The eluted fraction was concentrated by ultrafiltration through a filter with a nominal cut-off value of 10 kD, and purified further by size exclusion chromatography through a HiLoad™ 16/600 Superdex™ 75 prep grade column (GE Healthcare Biosciences, Piscataway, PA), equilibrated with 10 mM HEPES pH 7.5, 125 mM NaCl, and 1 mM TCEP. The protein appeared as a single peak at the expected range for the corresponding molecular weight. Fractions were collected and concentrated further to a protein stock of 150 μM. Total yield was about 3 mg per liter of culture.

5.2. Fluorescent polarization assay

A solution containing ~5 μM recombinant E. Coli-expressed fusion protein GST-eIF4E, 60 nM of the fluorescein labeled eIF4G-derived peptide KYTYDELFQLK (ε-Flu)-OH (KD = 3 μM), 0.05% bovine γ-globulin, and 2 mM DTT in a buffer composed of 50 mM sodium phosphate and 50 mM KCl at pH 6.5 is titrated with increasing concentrations (0.01–100 μM) of the competitive inhibitor for eIF4E and the decrease in fluorescence anisotropy is measured. The assay is carried out in black 384-well plates in triplicates and results in IC_{50}s.

5.3. Assessment of the disruption of eIF4E/eIF4G interaction by the m^7GTP pull-down assay

Melanoma CRL-2813 cell lines were incubated for 6 h in the presence of the small molecule, harvested by centrifugation, and lysed by multiple freeze–thaw cycles. Subsequently, the lysate was incubated with m^7GTP-Sepharose beads for 1 h at 4 °C to pull-down eIF4E. The beads were separated, extensively washed and the bound eIF4E and eluted with free m7GTP, the supernatant was separated by SDS-PAGE and Western blotted by polyclonal antibodies against 4E-BP1 and monoclonal antibodies against eIF4E and eIF4G.

5.4. Intrinsic tryptophan fluorescence intensity quenching

A stock solution of 0.1 μM GST-eIF4E in 100 mM sodium phosphate (pH 7.5) was prepared, 3 mL loaded into a 1 cm × 1 cm × 3 cm cuvette, and fluorescence measured with a PTI spectrofluorometer (Photon Technology International, Inc., Birmingham, NJ); The excitation light wavelength was set at 280 nm. Fluorescence intensity was recorded between 310 nm and 400 nm, with a 1 nm step each second, and 1 nm resolution. After the initial fluorescence of the protein alone was recorded the cuvette was washed. An appropriate amount of compound from a stock solution in DMSO was added and supplemented with DMSO to a final volume of 20 μL, 3 mL of the prepared protein stock solution was added, the sample was mixed and the fluorescence was measured again. This was repeated by the addition more of the tested compounds to attain different final concentrations until the fluorescence signal was almost completely quenched.

5.5. Western blot analysis

Western blot analysis was carried out as described [28]. Anti-eIF4E antibody (Cat. 610269) was from BD Transduction Laboratories (San Jose, CA); Anti-Survivin (Cat. 2808S), Anti-Cyclin E (Cat. 4129S), anti-4EBP-1 (Cat. 9452S), and anti-eIF4G (Cat. 2498S) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-Cyclin D1 (Cat. SC-8396), anti-Actin (Cat. SC-1615), and anti-α-Tubulin (Cat. SC-5546) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX).

5.6. Real time PCR

Total RNA was extracted with FastLane Cell One-Step Kit (Qiagen, Germantown, MD) and DNAse I treated according to manufacturer's recommendations. 1-Step Real-time PCR was performed on a Bio-Rad iCycler IQ5 system by using B-R 1-Step SYBR Green qRT-PCR Kit (Quanta BioSciences, Gaithersburg, MD) according to manufacturer's specifications. All primers were purchased from QuantiFect Primer Assay Database (Qiagen, Germantown, MD). All PCRs were performed in triplicate in at least two independent PCR runs. Mean values of these repeated measurements were used for calculation. To calibrate the results, all the transcript quantities were normalized to 18S rRNA (18S ribosomal RNA-like mRNA in mouse).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.034. These data include MOL files and InChiKeys of the most important compounds described in this article.

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