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Research paper

Novel 1,3-thiazolidin-4-one derivatives as promising anti-*Candida* agents endowed with anti-oxidant and chelating properties

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ABSTRACT

Pursuing our recent outcomes regarding the antifungal activity of *N*-substituted 1,3-thiazolidin-4-ones, we synthesized thirty-six new derivatives introducing aliphatic, cycloaliphatic and heteroaromatic moieties at N1-hydrazine connected with C2 position of the thiazolidinone nucleus and functionalizing the lactam nitrogen with differently substituted (NO_2, NH_2, Cl and F) benzyl groups. These compounds were tested to evaluate their minimum inhibitory concentration (MIC) against several clinical *Candida* spp. with respect to topical and systemic reference drugs (clotrimazole, fluconazole, ketoconazole, miconazole, tioconazole, amphotericin B). Moreover, anti-oxidant properties were also evaluated by using different protocols including free radical scavenging (DPPH and ABTS), reducing power (CUPRAC and FRAP), metal chelating and phosphomolybdenum assays. Moreover, for the most active derivatives we assessed the toxicity (CC_{50}) against Hep2 human cells in order to characterize them as multi-target agents for fungal infections.

1. Introduction

Commensal fungi like *Candida* species could become opportunistic pathogens causing infections whose severity depends on the state of health of the patients, ranging from the mucocutaneous mycosis to the more severe systemic candidiasis in those cases in which the immune system is compromised (AIDS, cancer, transplantation or broad spectrum antibiotic therapy) [1]. Recently, many cases of candidiasis provoked by *C. albicans* and non-*albicans* species such as *C. tropicalis* and *C. parapsilosis* have been reported [2–5] together with several data revealing that systemic mycoses due to *C. glabrata*, *C. krusei* and *C. sakè* are emerging [6–9]. Nowadays the therapy for these fungal infections is mainly based on the use of the drugs belonging to the class of azoles but, despite their safety profile with good tolerability and bioavailability, their use is leading to an increasing drug resistance and, for this reason, a compelling need to discover new drugs with a different mechanism of action is required. In this regard, new compounds from the class of thiazolidinones could be good candidates because of their anti-mycotic effects reported in many studies [10–17]. Recently, starting from the promising results of a scaffold of thiazol-2-yl-hydrazine derivatives designed by our group in the past [18–22], we reported the synthesis and the biological study of a large library of 4-thiazolidinones bearing (cyclo)aliphatic or (hetero)aryl moieties at N1-hydrazine and an aromatic ring at the lactam NH, demonstrating that these compounds possessed anti-*Candida* activity and most of them displayed low cytotoxicity against human cells [23]. On the basis of these interesting outcomes, we synthesized a new library of thirty-six new antifungal drugs (**1**–**36**), functionalizing the lactam nitrogen of the thiazolidinone nucleus using different benzyl bromides substituted with halogens (F and Cl), nitro and amino groups. At N1-hydrazine portion we selected four substituents which gave the best results from our previous works (2-propane, cyclopentane, 2-acetylthiophene and 2-acetylpyridine).

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0223-5234/

According to the established guidelines of Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST), we assessed the susceptibility of twenty-two clinical *Candida* spp. strains to our products with the determination of their minimum inhibitory concentration (MIC) [24]. Then, the most active compounds were assayed to evaluate their cell toxicity (CC_{50}) on Hep2 cells in order to define their selectivity against *Candida* spp. and we figured out that these derivatives were as cytotoxic as the well established reference drug (clotrimazole). In addition, we screened the best representative compounds for their anti-oxidant and chelating properties which could be ancillary for the treatment of fungal infections.

2. Chemistry

We designed thirty-six new derivatives structurally characterized by the 1,3-thiazolidin-4-one nucleus presenting at the N1-hydrazine four different moieties as the chemical requirements for the antimicrobial effect: an aliphatic chain (C_3), a five-membered cycloaliphatic ring (cyclopentane), a five-membered aromatic ring (thiophene), and a six-membered heteroaromatic nucleus (pyridine). These substituents were chosen on the basis of the best antifungal activity registered for this scaffold as previously reported [23].

The general synthesis of the target molecules is outlined in Scheme 1. Selected carbonyl compounds reacted directly with thiosemicarbazide in ethanol using acetic acid as the catalyst. The resulting thiosemicarbazones reacted with ethyl bromoacetate in methanol and sodium acetate in order to obtain the 1,3-thiazolidin-4-one derivatives [25,26]. Then, the reaction between the thiazolidinones and 2-/3-nitrobenzyl bromide, 2-/3-/4-chlorobenzyl bromide and 2-/3-/4-fluorobenzyl bromide in anhydrous acetone and potassium carbonate gave the *N*-functionalized thiazolidinone derivatives (**1**–**32**). 4-Aminobenzyl derivatives (**33**–**36**) were obtained by the reduction of *N*-substituted-4-thiazolidinones bearing a *para*-nitrobenzyl at lactam nitrogen [23]; sodium dithionite was previously solubilized in a basic aqueous solution and added dropwise to a stirring suspension of the 4-nitrobenzyl compounds in tetrahydrofuran at room temperature. All the synthesized compounds were purified by column chromatography before characterization by spectroscopic methods (IR and ^1H and ^{13}C NMR) and elemental analysis to ensure their purity.

3. Antibacterial and anti-*Candida* activities

Firstly, derivatives **1**–**36**, dissolved in dimethylsulfoxide (DMSO), were evaluated for their antibacterial activity. Organisms from routine clinical Gram-positive (*Staphylococcus aureus, Staphylococcus warneri, Streptococcus faecalis, Streptococcus α-hemolyticus*) and Gram-negative isolates (*Escherichia coli, Proteus mirabilis, Enterobacter* spp., *Klebsiella oxytoca*) from the respiratory tract were carefully collected from specimens of patients at the Hospital 'Azienda Policlinico Umberto I°' (Sapienza University of Rome, Italy). The isolates were subcultured on a qualified medium to ensure purity. The isolates were identified by conventional methodologies; all isolates were subcultured to ensure optimal growth. The *in vitro* antibacterial activities of the compounds were determined by the broth microdilution method with Mueller-Hinton II broth (BBL Microbiology Systems, Cockeysville, MD), as strictly recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [27].

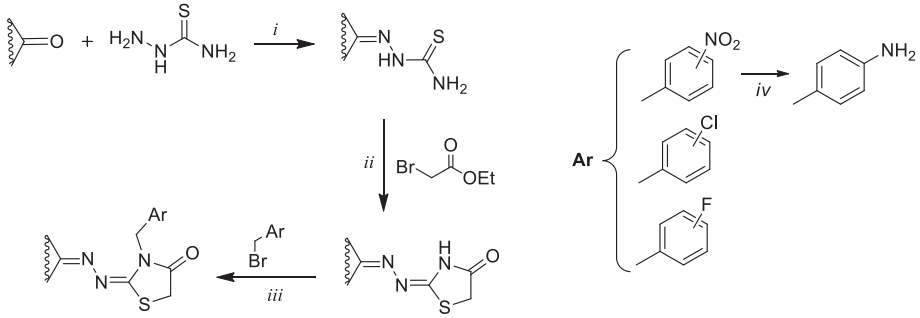
Once verified the absence of antibacterial effects, their antifungal activity was evaluated against twenty-two clinical fungal isolates of *Candida* spp. (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. sakè*) and compared with six topical and systemic reference drugs clotrimazole, fluconazole, ketoconazole, miconazole, tioconazole and amphotericin B (Table 1). The used clinical isolates were recently collected from specimens of patients at the 'Azienda Policlinico Umberto I°' (Sapienza University of Rome) and were obtained from haematology/oncology and surgery departments, which also included an intensive care unit. In particular, the samples were isolated from the upper and lower respiratory tract, blood, and indwelling venous catheters; the isolates were identified by conventional methodologies. Prior to testing, each isolate was subcultured on a qualified medium to ensure purity and optimal growth. All derivatives were dissolved in DMSO. The *in vitro* antifungal activities were determined by the broth microdilution method with Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, MD) as recommended by the NCCLS [28].

4. Cell toxicity

Compounds **3**, **5**, **10**, **11**, **15**, **19**, **20**, **27**, **28**, **30** and **33**, endowed with the strongest antifungal effect against *Candida* spp., were also assayed at five concentrations ranging from 0.05 to 100 μg/mL to evaluate their cytotoxic activity against a cultured cell line derived from a human laryngeal epidermoid carcinoma (Hep2) with respect to the reference drug (clotrimazole), as reported in Table 2. Cell viability was analyzed using Trypan Blue exclusion test [29].

5. Anti-oxidant and metal chelating assays

A large number of the most representative derivatives were also tested for their anti-oxidant and chelating properties in order to assess ancillary activities which could be useful to obtain multi-target agents against *Candida* infections (Table 3). The chelating



Scheme 1. Synthesis of derivatives **1**–**36**. Reagents and conditions: (*i*) EtOH, acetic acid (cat.), rt; (ii) MeOH, CH_3COONa, rt; (*iii*) anhydrous acetone, anhydrous K_2CO_3, reflux; (*iv*) if 4-NO_2 substituted: $Na_2S_2O_4$, $NaHCO_3$, water, THF, rt.

Table 1
Minimal inhibitory concentration (MIC) expressed as geometric mean of derivatives **1**–**36** and reference compounds against twenty-two clinical strains of *Candida* species.

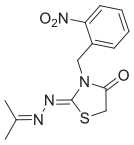
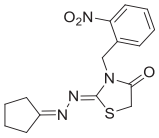
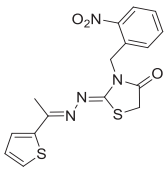
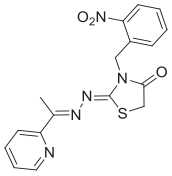
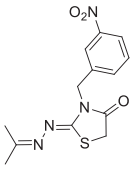
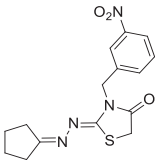
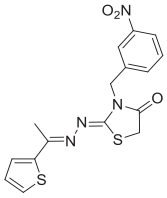
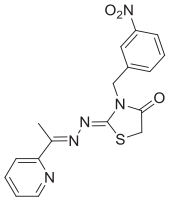
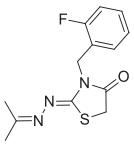
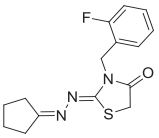
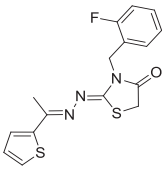
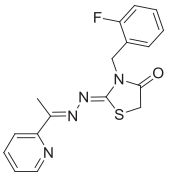
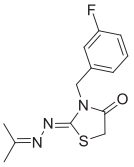
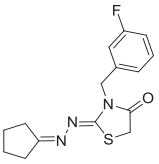
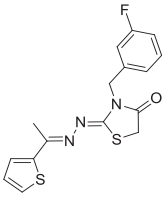
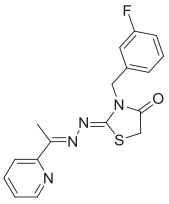
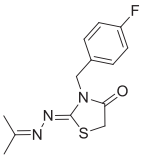
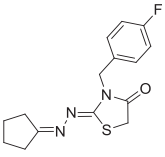
Compound	Structure	Tested fungi (MIC μg/mL)					
		C. albicans (8)	*C. glabrata* (4)	*C. tropicalis* (3)	*C. krusei* (3)	*C. parapsilosis* (2)	*C. sakè* (2)
1		2	4	8	2	16	4
2		4	2	8	4	16	4
3		2	2	4	2	8	2
4		2	2	4	4	16	2
5		2	2	2	2	16	2
6		4	16	4	8	4	2
7		4	2	4	2	8	4
8		4	2	8	8	8	4
9		8	2	4	8	4	8

Table 1 (*continued*)

Compound	Structure	Tested fungi (MIC μg/mL)					
		C. albicans (8)	*C. glabrata* (4)	*C. tropicalis* (3)	*C. krusei* (3)	*C. parapsilosis* (2)	*C. sakè* (2)
10		2	2	2	8	2	2
11		2	4	2	4	4	2
12		4	8	4	4	4	8
13		4	4	4	4	2	8
14		4	2	16	2	4	8
15		2	8	2	2	2	2
16		2	2	8	8	2	4
17		8	4	16	16	2	8
18		8	4	16	8	2	8

(*continued on next page*)

Table 1 (*continued*)

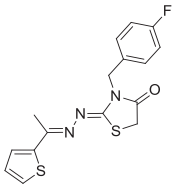
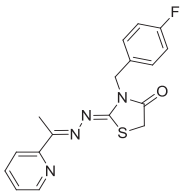
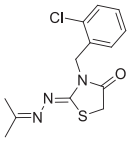
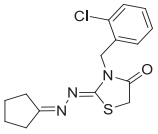
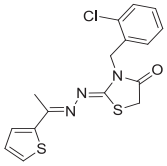
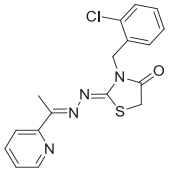
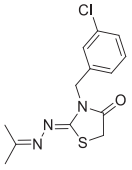
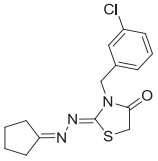
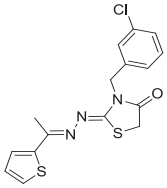
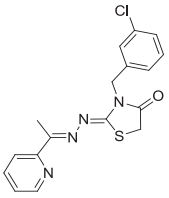
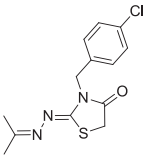
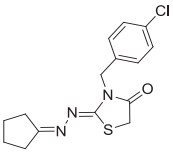
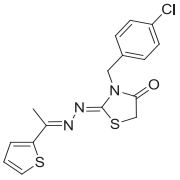
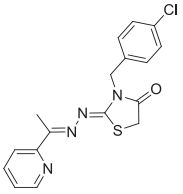
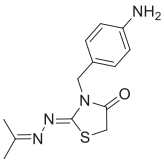
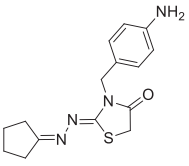
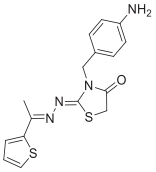
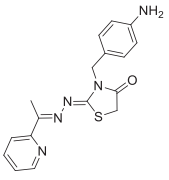
Compound	Structure	Tested fungi (MIC μg/mL)					
		C. albicans (8)	*C. glabrata* (4)	*C. tropicalis* (3)	*C. krusei* (3)	*C. parapsilosis* (2)	*C. sakè* (2)
19		2	2	2	2	4	8
20		2	2	2	8	4	2
21		2	2	16	8	8	2
22		2	4	4	8	8	2
23		4	4	2	8	8	8
24		4	4	2	4	8	4
25		8	4	2	2	4	4
26		4	4	8	16	4	2
27		2	2	2	2	2	2

Table 1 (*continued*)

Compound	Structure	Tested fungi (MIC μg/mL)					
		C. albicans (8)	*C. glabrata* (4)	*C. tropicalis* (3)	*C. krusei* (3)	*C. parapsilosis* (2)	*C. sakè* (2)
28		2	2	2	4	2	16
29		4	2	8	8	2	4
30		2	2	2	8	2	4
31		4	2	8	8	4	8
32		8	4	8	8	4	8
33		4	1	32	2	2	4
34		2	16	32	16	64	256
35		4	8	2	8	8	2
36		2	8	2	8	8	2
Fluconazole		2	2	2	2	2	2

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	Tested fungi (MIC μg/mL)					
		C. albicans (8)	*C. glabrata* (4)	*C. tropicalis* (3)	*C. krusei* (3)	*C. parapsilosis* (2)	*C. sakè* (2)
Ketoconazole		2	2	2	4	2	4
Clotrimazole		2	2	2	2	2	2
Miconazole		2	4	2	4	4	2
Tioconazole		8	4	8	8	8	4
Amphotericin B		4	4	2	4	2	2

Table 2
Cytotoxic effect (CC_{50}) of the most active compounds and clotrimazole tested on Hep2 cells after 24 h of incubation at 37 °C using Trypan blue exclusion test expressed as cell survival fraction (%).[a]

Compound	0.05 μg/mL	0.5 μg/mL	5 μg/mL	10 μg/mL	50 μg/mL	CC_{50} (μg/mL)
3	88.00%	84.00%	74.00%	61.00%	0.00%	20
5	79.00%	68.00%	51.00%	0.00%	0.00%	5
10	82.00%	69.00%	52.00%	37.00%	0.00%	11
11	91.00%	80.00%	62.00%	41.00%	0.00%	15
15	80.00%	68.00%	42.00%	0.00%	0.00%	6
19	83.00%	76.00%	68.00%	52.00%	0.00%	15
20	91.00%	83.00%	70.00%	42.00%	0.00%	16
27	94.00%	89.00%	82.00%	63.00%	0.00%	21
28	94.00%	87.5%	85.00%	69.00%	0.00%	21
30	89.00%	83.00%	59.00%	47.00%	0.00%	16
Clotrimazole	93.00%	93.40%	92.50%	68.00%	10.20%	18

[a] Cells incubated with culture medium alone represented the controls. Viability with 2 μL of DMSO = 94%, viability with 20 μL of DMSO = 87%. Data represent arithmetic means of at least three independent experiments.

Table 3
Anti-oxidant and chelating properties of the most active and representative compounds.

Compound	Anti-oxidant assays (mM)					Chelating activity (mM)
	Phosphomolybdenum assay (EC_{50})	FRAP (EC_{50})	CUPRAC (EC_{50})	DPPH (IC_{50})	ABTS (IC_{50})	IC_{50}
3	1.12 ± 0.03	2.40 ± 0.01	2.25 ± 0.02	>5	18.07 ± 1.24	>5
5	1.24 ± 0.02	5.29 ± 0.27	2.35 ± 0.03	>5	19.36 ± 1.59	>5
10	0.70 ± 0.03	5.63 ± 0.17	2.37 ± 0.03	>5	12.43 ± 0.14	>5
11	1.26 ± 0.06	2.13 ± 0.03	2.40 ± 0.04	>5	15.69 ± 0.84	3.84 ± 0.16
15	1.36 ± 0.02	2.62 ± 0.05	2.55 ± 0.02	>5	21.13 ± 2.28	4.64 ± 0.47
19	1.37 ± 0.02	2.02 ± 0.09	1.85 ± 0.14	>5	21.54 ± 3.24	3.90 ± 0.04
20	1.16 ± 0.06	6.22 ± 0.23	1.75 ± 0.02	>5	16.15 ± 0.12	3.67 ± 0.23
27	1.16 ± 0.03	2.32 ± 0.08	1.59 ± 0.01	>5	20.32 ± 3.42	1.64 ± 0.03
28	2.04 ± 0.10	4.93 ± 0.42	1.40 ± 0.06	>5	26.79 ± 2.02	2.76 ± 0.03
30	0.56 ± 0.04	4.80 ± 0.21	2.10 ± 0.10	>5	18.31 ± 1.59	>5
33	1.20 ± 0.01	2.76 ± 0.13	1.77 ± 0.01	>5	2.04 ± 0.04	>5
Trolox	1.13 ± 0.05	0.16 ± 0.01	0.24 ± 0.02	0.40 ± 0.01	0.66 ± 0.02	–
EDTA	–	–	–	–	–	0.02 ± 0.001

property has been studied by using EDTA as reference compound, whereas the anti-oxidant activity has been evaluated by five experimental and well established procedures with respect to Trolox as reference compound following the promising data obtained for some thiazolidinone-based compounds by other research groups [30].

The phosphomolybdenum assay is based on the reduction of Mo(VI) to green phosphate/Mo(V) by antioxidant compounds, whereas the free radical scavenging activities were evaluated by DPPH and ABTS assays. Antioxidant compounds usually interact with free radicals by electron or hydrogen atom transfer, thus converting them into stable and non-radical molecules. Generally, the radical scavenging activities followed a similar trend in this scaffold. Antioxidant properties and reducing power are strongly related by breaking free radical chain reaction and thus inhibiting lipid peroxidation. Reducing power was evaluated by the transformation of Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+} in the presence of the tested compounds. By analyzing the data of their metal chelating activity in Table 3, it is also possible to state the ability of this scaffold in the prevention of reactive oxygen species production. These findings support that chelation of transition metals is one of the ways through which thiazolidinones exert their antioxidant activity.

6. Results and discussion

A new class of *N*-functionalized-1,3-thiazolidin-4-one derivatives were easily synthesized in high yields and evaluated *in vitro* for their antimicrobial effect against several bacterial and fungal clinical isolates. None of the products possessed antibacterial activity (MIC >256 μg/mL, see Supplementary material); consequently the compounds have shown to be effective selectively against fungal pathogens. As reported in Table 1 for the *in vitro* antimycotic activity assay many compounds had good antifungal effects towards *C. albicans* and non-*albicans* spp. with MIC values that were similar or better than those of reference drugs. Among the aliphatic derivatives, compound **5**, provided with a 3-nitrobenzyl ring at the lactam NH, was the most active with MIC values of 2 μg/mL against all the species, except for *C. parapsilosis*,

while compound **33**, with an amino group at C4 of the aromatic substituent, was active against *C. krusei* and *C. parapsilosis* (MICs = 2 μg/mL) and especially against *C. glabrata* (MIC = 1 μg/mL). Also the cyclopentane derivative **10**, bearing a fluorine at C2 of the benzyl ring, was very active against *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. sakè* (MICs = 2 μg/mL), whereas the 4-chlorobenzyl compound **30** was very effective against *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* (MICs = 2 μg/mL). Among the thiophene derivatives, compounds **3**, **11**, **15** and **19** possessed a MIC of 2 μg/mL against *C. albicans* and compound **27**, in which the benzyl group was functionalized with a chlorine in the *meta* position, was shown to be very active against all the tested fungal species (MIC = 2 μg/mL). Finally, for the pyridine derivatives, we found that compounds **20** and **28**, with a 4-fluoro and 3-chlorobenzyl groups at the lactam nitrogen respectively, displayed MIC values of 2 μg/mL against most of *Candida* species. With respect to the previous series of thiazolidinones [23], the introduction of specific electron-withdrawing substituents on the benzyl group kept the antifungal activity similar to that of reference drugs.

In consideration of these results, we evaluated the cytotoxic effects (CC_{50}) by Trypan blue exclusion test at five different concentrations of the most active derivatives, possessing the lowest MIC values against *C. albicans* and other *Candida* spp. (**3**, **5**, **10**, **11**, **15**, **19**, **20**, **27**, **28**, **30** and **33**), and of the reference drug clotrimazole (Table 2). All the selected compounds have proven to be as cytotoxic as clotrimazole and to display cytotoxicity at higher concentration than that required for the pharmacological activity.

Moreover, a large number of compounds displayed similar antioxidant activity of Trolox in the Phosphomolybdenum assay with EC_{50} values in the millimolar range, except for derivatives **10** and **30** both characterized by the cyclopentane ring and a halogen on the aryl group (EC_{50} values in the micromolar range). They resulted less active than the reference compound in the other four anti-oxidant assays (FRAP, CUPRAC, DPPH and ABTS) showing the same biological trend. As regards the chelating activity, only few derivatives were endowed with discrete IC_{50} values with respect to EDTA and they highlight the beneficial role of a thiophene ring in the structure (**11**, **15**, **19** and **27**). In conclusion, we synthesized a new scaffold of *N*-benzylated thiazolidinone derivatives possessing antimycotic activity against *Candida* spp. and endowed with limited cytotoxicity and promising anti-oxidant and chelating activity.

7. Experimental section

The chemicals, solvents for synthesis, and spectral grade solvents were purchased from Aldrich (Italy) and used without further purification. Melting points (uncorrected) were determined automatically on an FP62 apparatus (Mettler-Toledo). ^1H and ^{13}C NMR spectra were recorded at 400 and 101 MHz, respectively, on a Bruker spectrometer using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts are expressed as δ units (parts per millions) relative to the solvent peak. Coupling constants *J* are valued in Hertz (Hz). IR spectra (neat) were registered on a Perkin Elmer FT-IR Spectrometer Spectrum 1000. Elemental analyses for C, H, and N were recorded on a Perkin–Elmer 240 B microanalyzer and the analytical results were within ±0.4% of the theoretical values for all compounds. All reactions were monitored by TLC performed on 0.2 mm thick silica gel plates (60 F_{254} Merck).

In general, the IR spectrum (neat) for derivatives **1**–**36** showed a band at about 3027 cm^{-1} (C_{sp2}-H stretching), at about 1693 (C=O stretching), at about 1622 (C=N stretching), and at about 1582 and 1447 (C=C stretching).

7.1. General procedure for the synthesis of compounds 1-36

The initial carbonyl compound (50 mmol) was dissolved/suspended in ethanol (50 mL) and magnetically stirred with thiosemicarbazide (50 mmol) and catalytic amounts of acetic acid for 8–24 h at room temperature. The obtained thiosemicarbazone was filtered, washed with appropriate solvent (*n*-hexane. petroleum ether or diethyl ether) and dried under vacuum. The intermediate thiosemicarbazone (50 mmol) reacted with ethyl-bromoacetate (50 mmol), in methanol (50 mL) and sodium acetate (50 mmol) at room temperature under magnetic stirring for 24 h. The resulting 4-thiazolidinone was poured on ice, filtered or extracted with chloroform and purified by column chromatography (SiO_2. ethyl acetate/*n*-hexane 1/2). Then, the resulting thiazolidinone (50 mmol) was dissolved/suspended in 50 mL of anhydrous acetone in the presence of anhydrous potassium carbonate (50 mmol), and reacted with equimolar amounts of 2-, 3-nitrobenzyl bromide, 2-, 3-, 4-chlorobenzyl bromide and 2-, 3-, 4-fluorobenzyl bromide for 24–48 h. The products were poured on ice, filtered or extracted with chloroform (3 × 50 mL) and purified by column chromatography (SiO_2. ethyl acetate/*n*-hexane) in order to obtain the title compounds in high yields. To obtain 4-aminobenzyl derivatives (33–36), sodium dithionite (5.5 eq) was solubilized in a solution of water (30 mL) and sodium bicarbonate (5.5 eq), then the aqueous solution was added dropwise to a stirring suspension of 4-nitrobenzyl compounds (1.0 eq) in tetrahydrofuran (50 mL) at room temperature for 2 h. Then tetrahydrofuran was evaporated *in vacuo* and the solid precipitated from the aqueous phase was filtered and washed with water and petroleum ether to give the desired amino derivative.

7.1.1. 3-(2-Nitrobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**1**)

White powder, mp 144–146 °C, 91% yield; ^1H NMR (400 MHz, $CDCl_3$): δ 1.84 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 3.89 (s, 2H, CH_2, thiazolidinone), 5.40 (s, 2H, $ArCH_2$), 7.31 (bs, 1H, Ar), 7.43–7.47 (m, 1H, Ar), 7.56–7.60 (m, 1H, Ar), 8.06–8.08 (d, J = 8.0 Hz, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.36 (CH_3), 24.95 (CH_3), 39.09 (CH_2, Thiaz.), 42.80 (CH_2), 125.18 (Ar), 128.67 (Ar), 129.15 (Ar), 131.25 (Ar), 134.44 (Ar), 148.67 (Ar), 159.46 (C=N, Thiaz.), 165.79 (C=N), 172.75 (C=O). Anal. Calcd. for $C_{13}H_{14}N_4O_3S$: C, 50.97; H, 4.61; N, 18.29. Found: C, 50.78; H, 4.30; N, 18.02.

7.1.2. 3-(2-Nitrobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**2**)

Grey powder, mp 179–180 °C, 87% yield; ^1H NMR (400 MHz, DMSO-d_6): δ 1.74–1.75 (m, 4H, cyclopentane), 2.25–2.27 (m, 2H, cyclopentane), 2.46–2.47 (m, 2H, cyclopentane), 3.89 (s, 2H, CH_2, thiazolidinone), 5.38 (s, 2H, $ArCH_2$), 7.31–7.33 (m, 1H, Ar), 7.43–7.47 (t, 1H, Ar), 7.56–7.60 (t, 1H, Ar), 8.06–8.08 (d, J = 8.8 Hz, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.44 (CH_2), 24.76 (CH_2), 29.86 (CH_2), 32.59 (CH_2, Thiaz.), 32.97 (CH_2), 42.71 (CH_2), 125.13 (Ar), 128.80 (Ar), 129.16 (Ar), 131.27 (Ar), 134.44 (Ar), 148.73 (C=N, Thiaz.), 159.22 (C=N), 172.69 (Ar), 178.39 (C=O). Anal. Calcd. for $C_{15}H_{16}N_4O_3S$: C, 54.20; H, 4.85; N, 16.86. Found: C, 54.44; H, 4.70; N, 17.08.

7.1.3. 3-(2-Nitrobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**3**)

Pink powder, mp 150–151 °C, 93% yield; ^1H NMR (400 MHz, $CDCl_3$): δ 2.26 (s, 3H, CH_3), 3.90 (s, 2H, CH_2, thiazolidinone), 5.44 (s, 2H, $ArCH_2$), 7.05–7.06 (m, 1H, thiophene), 7.32–7.48 (m, 4H, thiophene + Ar), 7.59–7.61 (m, 1H, Ar), 8.07–8.09 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.98 (CH_3), 32.80 (CH_2, Thiaz.), 42.99 (CH_2), 125.23 (Ar), 128.20 (Thioph.), 128.88 (Ar), 129.25 (Ar), 129.32

(Thioph.), 130.06 (Thioph.), 131.29 (Ar), 134.50 (Ar), 143.32 (Thioph.), 148.75 (Ar), 158.33 (C=N, Thiaz.), 161.96 (C=N), 172.85 (C=O). Anal. Calcd. for $C_{16}H_{14}N_4O_3S_2$: C, 51.32; H, 3.77; N, 14.96. Found: C, 51.65; H, 3.50; N, 15.11.

*7.1.4. 3-(2-Nitrobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**4**)*

Grey powder, mp 170–171 °C. 86% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.37 (s, 3H, CH_3), 3.94 (s, 2H, CH_2, thiazolidinone), 5.50 (s, 2H, $ArCH_2$), 7.35–7.39 (m, 2H, Ar), 7.45–7.50 (m, 1H, Ar), 7.60 (bs, 1H, Ar), 7.76–7.77 (m, 1H, Ar), 8.10–8.12 (m, 1H, Ar), 8.16–8.20 (m, 1H, Ar), 8.65 (bs, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.72 (CH_3), 32.88 (CH_2, Thiaz.), 43.13 (CH_2), 120.91 (Pyr), 125.13 (Pyr), 125.27 (Ar), 128.87 (Ar), 129.28 (Ar), 131.21 (Ar), 134.51 (Ar), 137.08 (Pyr), 148.73 (Ar), 149.33 (Pyr), 155.32 (Pyr), 162.40 (C=N, Thiaz.), 163.69 (C=N), 172.91 (C=O). Anal. Calcd. for $C_{17}H_{15}N_5O_3S$: C, 55.27; H, 4.09; N, 18.96. Found: C, 55.10; H, 4.21; N, 19.12.

*7.1.5. 3-(3-Nitrobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**5**)*

White powder, mp 190–191 °C, 92% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.08 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 3.82 (s, 2H, CH_2, thiazolidinone), 5.05 (s, 2H, $ArCH_2$), 7.50–7.54 (t, 1H, Ar), 7.80–7.82 (d, J = 7.6 Hz, 1H, Ar), 8.17–8.19 (d, J = 7.6 Hz, 1H, Ar), 8.39 (s, 1H, Ar). ^{13}C NMR (101 MHz, $CDCl_3$) δ 18.83 (CH_3), 25.08 (CH_3), 32.33 (CH_2, Thiaz.), 45.73 (CH_2), 123.06 (Ar), 124.25 (Ar), 129.53 (Ar), 135.10 (Ar), 137.60 (Ar), 148.12 (Ar), 158.93 (C=N, Thiaz.), 166.97 (C=N), 171.80 (C=O). Anal. Calcd. for $C_{13}H_{14}N_4O_3S$: C, 50.97; H, 4.61; N, 18.29. Found: C, 50.71; H, 4.88; N, 18.41.

*7.1.6. 3-(3-Nitrobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**6**)*

White powder, mp 90–95 °C, 90% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.81–1.85 (m, 4H, cyclopentane), 2.51–2.56 (m, 4H, cyclopentane), 3.82 (s, 2H, CH_2, thiazolidinone), 5.03 (s, 2H, $ArCH_2$), 7.52–7.54 (t, 1H, Ar), 7.81–7.83 (m, 1H, Ar), 8.17–8.19 (m, 1H, Ar), 8.40 (s, 1H, Ar). ^{13}C NMR (101 MHz, $CDCl_3$) δ 24.67 (CH_2), 24.92 (CH_2), 30.53 (CH_2), 32.39 (CH_2, Thiaz.), 33.44 (CH_2), 45.69 (CH_2), 123.09 (Ar), 124.25 (Ar), 124.49 (Ar), 129.50 (Ar), 129.57 (Ar), 148.10 (Ar), 158.48 (C=N, Thiaz.), 171.70 (C=N), 180.22 (C=O). Anal. Calcd. for $C_{15}H_{16}N_4O_3S$: C, 54.20; H, 4.85; N, 16.86. Found: C, 54.02; H, 4.63; N, 16.59.

*7.1.7. 3-(3-Nitrobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**7**)*

Yellow powder, mp 165–170 °C, 82% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.36 (s, 3H, CH_3), 4.05 (s, 2H, CH_2, thiazolidinone), 5.05 (s, 2H, $ArCH_2$), 7.11–7.13 (m, 1H, thiophene), 7.54–7.55 (m, 1H, thiophene), 7.61–7.62 (m, 1H, thiophene), 7.65–7.69 (m, 1H, Ar), 7.83–7.85 (m, 1H, Ar), 8.17–8.19 (m, 1H, Ar), 8.30 (s, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.34 (CH_3), 32.71 (CH_2, Thiaz.), 45.69 (CH_2), 123.14 (Ar), 123.61 (Ar), 128.22 (Thioph.), 129.35 (Thioph.), 130.06 (Thioph.), 130.50 (Ar), 135.34 (Ar), 138.63 (Ar), 143.36 (Thioph.), 148.15 (Ar), 158.39 (C=N, Thiaz.), 162.27 (C=N), 172.73 (C=O). Anal. Calcd. for $C_{16}H_{14}N_4O_3S_2$: C, 51.32; H, 3.77; N, 14.96. Found: C, 51.19; H, 3.95; N, 15.16.

*7.1.8. 3-(3-Nitrobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**8**)*

Grey powder, mp 195–200 °C, 94% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.42 (s, 3H, CH_3), 4.10 (s, 2H, CH_2, thiazolidinone), 5.09 (s, 2H, $ArCH_2$), 7.45 (bs, 1H, Ar), 7.68 (bs, 1H, Ar), 7.86–7.88 (m, 2H, Ar), 8.05–8.07 (m, 1H, Ar), 8.17–8.19 (m, 1H, Ar), 8.32 (s, 1H, Ar), 8.64 (s, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.05 (CH_3), 32.77 (CH_2, Thiaz.), 45.79 (CH_2), 120.95 (Pyr), 123.18 (Ar), 123.57 (Ar), 125.15 (Pyr), 130.54 (Ar), 135.40 (Ar), 137.13 (Pyr), 138.57 (Ar), 148.20 (Ar), 149.37 (Pyr), 155.34 (Pyr), 163.72 (C=N, Thiaz.), 164.08 (C=N), 172.83 (C=O). Anal. Calcd. for $C_{17}H_{15}N_5O_3S$: C, 55.27; H, 4.09; N, 18.96. Found: C, 55.44; H, 4.32; N, 19.20.

*7.1.9. 3-(2-Fluorobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**9**)*

Yellow powder, mp 73–75 °C, 93% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.99 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 3.87 (s, 2H, CH_2, thiazolidinone), 5.06 (s, 2H, $ArCH_2$), 7.05–7.11 (m, 2H, Ar), 7.29–7.32 (m, 2H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.54 (CH_3), 24.93 (CH_3), 32.35 (CH_2, Thiaz.), 39.10 (CH_2), 110.00 (Ar), 115.70 (d, $J_{C\text{-}F}$ = 29.3 Hz, Ar), 123.33 (d, $J_{C\text{-}F}$ = 20.0 Hz, Ar), 124.83 (Ar), 129.91 (d, $J_{C\text{-}F}$ = 13.3 Hz, Ar), 159.55 (C=N, Thiaz.), 165.73 (C=N), 172.43 (C=O). Anal. Calcd. for $C_{13}H_{14}FN_3OS$: C, 55.90; H, 5.05; N, 15.04. Found: C, 55.72; H, 4.78; N, 15.22.

*7.1.10. 3-(2-Fluorobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**10**)*

Brown oil, 64% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.76–1.80 (m, 4H, cyclopentane), 2.42–2.45 (m, 2H, cyclopentane), 2.56–2.57 (m, 2H, cyclopentane), 3.86 (s, 2H, CH_2, thiazolidinone), 5.06 (s, 2H, $ArCH_2$), 7.03–7.11 (m, 2H, Ar), 7.27–7.35 (m, 2H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.34 (CH_2), 24.89 (CH_2), 30.32 (CH_2), 32.66 (CH_2, Thiaz.), 33.16 (CH_2), 45.55 (CH_2), 114.23 (d, $J_{C\text{-}F}$ = 20.9 Hz, Ar), 115.12 (d, $J_{C\text{-}F}$ = 23.1 Hz, Ar), 123.89 (d, $J_{C\text{-}F}$ = 2.8 Hz, Ar), 130.43 (Ar), 139.11 (Ar), 158.11 (C=N, Thiaz.), 160.14 (C=N), 162.09 (d, $J_{C\text{-}F}$ = 244.7 Hz, Ar), 172.50 (C=O). Anal. Calcd. for $C_{15}H_{16}FN_3OS$: C, 59.00; H, 5.28; N, 13.76. Found: C, 59.25; H, 5.04; N, 13.99.

*7.1.11. 3-(2-Fluorobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**11**)*

Orange powder, mp 83–85 °C, 91% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.28 (s, 3H, CH_3), 4.06 (s, 2H, CH_2, thiazolidinone), 4.98 (s, 2H, $ArCH_2$), 7.10–7.21 (m, 3H, thiophene), 7.33–7.36 (m, 2H, Ar), 7.51–7.52 (m, 1H, Ar), 7.61–7.62 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.15 (CH_3), 32.60 (CH_2, Thiaz.), 115.76 (d, $J_{C\text{-}F}$ = 21.2 Hz, Ar), 123.26 (d, $J_{C\text{-}F}$ = 14.4 Hz, Ar), 124.86 (Ar), 128.19 (Thioph.), 129.24 (Thioph.), 129.94 (Ar), 129.99 (Thioph.), 130.09 (Ar), 143.37 (Ar), 158.27 (C=N, Thiaz.), 160.54 (d, $J_{C\text{-}F}$ = 246.7 Hz, Ar), 162.02 (C=N), 172.53 (C=O). Anal. Calcd. for $C_{16}H_{14}FN_3OS_2$: C, 55.31; H, 4.06; N, 12.09. Found: C, 55.60; H, 3.83; N, 12.21.

*7.1.12. 3-(2-Fluorobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**12**)*

Orange powder, mp 139–141 °C, 84% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.58 (s, 3H, CH_3), 3.90 (s, 2H, CH_2, thiazolidinone), 5.16 (s, 2H, $ArCH_2$), 7.06–7.14 (m, 2H, Ar), 7.38–7.49 (m, 3H, Ar), 7.91–7.92 (m, 1H, Ar), 8.26–8.29 (m, 1H, Ar), 8.74–8.77 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.89 (CH_3), 32.64 (CH_2, Thiaz.), 39.09 (CH_2), 114.90 (d, $J_{C\text{-}F}$ = 20.9 Hz, Ar), 115.77 (d, $J_{C\text{-}F}$ = 24.6 Hz, Ar), 120.91 (Pyr), 124.98 (d, $J_{C\text{-}F}$ = 24.7 Hz, Ar), 130.12 (Pyr), 137.09 (Ar), 139.24 (Ar), 139.24 (Pyr), 149.32 (Pyr), 155. 75 (Pyr), 158.34 (Ar), 158.87 (C=N, Thiaz.), 163.80 (C=N), 172.60 (C=O). Anal. Calcd. for $C_{17}H_{15}FN_4OS$: C, 59.63; H, 4.42; N, 16.36. Found: C, 59.36; H, 4.13; N, 16.61.

*7.1.13. 3-(3-Fluorobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**13**)*

Yellow oil, 68% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.06 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 3.81 (s, 2H, CH_2, thiazolidinone), 4.95 (s, 2H, $ArCH_2$), 6.97–7.01 (m, 1H, Ar), 7.14–7.23 (m, 2H, Ar), 7.26–7.32 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.64 (CH_3), 24.92 (CH_3), 32.39 (CH_2, Thiaz.), 45.66 (CH_2), 114.66 (d, $J_{C\text{-}F}$ = 10.8 Hz, Ar), 124.31 (d, $J_{C\text{-}F}$ = 3.1 Hz, Ar), 130.68 (d, $J_{C\text{-}F}$ = 4.6 Hz, Ar), 139.37 (d, $J_{C\text{-}$

$_F$ = 10.8 Hz, Ar), 158.71 (Ar), 159.87 (C=N, Thiaz.), 160.89 (Ar), 165.62 (C=N), 172.56 (C=O). Anal. Calcd. for $C_{13}H_{14}FN_3OS$: C, 55.90; H, 5.05; N, 15.04. Found: C, 55.75; H, 4.82; N, 14.89.

*7.1.14. 3-(3-Fluorobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**14**)*

White powder, mp 97–100 °C, 95% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.82–1.83 (m, 4H, cyclopentane), 2.51–2.54 (m, 4H, cyclopentane), 3.80 (s, 2H, CH_2, thiazolidinone), 4.93 (s, 2H, $ArCH_2$), 7.00–7.01 (m, 1H, Ar), 7.18–7.25 (m, 3H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.55 (CH_2), 24.84 (CH_2), 30.34 (CH_2), 32.43 (CH_2, Thiaz.), 33.02 (CH_2) 45.69 (CH_2), 114.82 (d, $J_{C\text{-}F}$ = 21.11 Hz, Ar), 115.30 (d, $J_{C\text{-}F}$ = 21.9 Hz, Ar), 124.53 (d, $J_{C\text{-}F}$ = 2.7 Hz, Ar), 130.82 (d, $J_{C\text{-}F}$ = 8.3 Hz, Ar), 139.33 (d, $J_{C\text{-}F}$ = 7.5 Hz, Ar), 158.43 (C=N, Thiaz.), 159.58 (C=N), 162.47 (d, $J_{C\text{-}F}$ = 244.5 Hz, Ar), 172.53 (C=O). Anal. Calcd. for $C_{15}H_{16}FN_3OS$: C, 59.00; H, 5.28; N, 13.76. Found: C, 59.18; H, 5.47; N, 13.50.

*7.1.15. 3-(3-Fluorobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**15**)*

Yellow powder, mp 84–85 °C, 89% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.33 (s, 3H, CH_3), 4.05 (s, 2H, CH_2, thiazolidinone), 4.93 (s, 2H, $ArCH_2$), 7.12–7.22 (m, 4H, thiophene + Ar), 7.38–7.41 (m, 1H, Ar), 7.53 (bs, 1H, Ar), 7.61–7.63 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.301 (CH_3), 32.66 (CH_2, Thiaz), 45.84 (CH_2), 114.75 (Ar), 115.02 (Ar), 124.13 (Ar), 124.35 (Ar), 128.20 (Thioph.), 129.25 (Thioph.), 130.00 (Thioph.), 130.84 (Ar), 139.35 (Ar), 143.39 (Thioph.), 158.24 (C=N, Thiaz.), 162.26 (C=N), 162.54 (d, $J_{C\text{-}F}$ = 245.0 Hz, Ar), 172.72 (C=O). Anal. Calcd. for $C_{16}H_{14}FN_3OS_2$: C, 55.31; H, 4.06; N, 12.09. Found: C, 55.56; H, 3.79; N, 11.84.

*7.1.16. 3-(3-Fluorobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**16**)*

Orange powder, mp 130–132 °C, 80% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.64 (s, 3H, CH_3), 3.86 (s, 2H, CH_2, thiazolidinone), 5.05 (s, 2H, $ArCH_2$), 7.00–7.03 (m, 1H, Ar), 7.20–7.35 (m, 3H, Ar), 7.44–7.46 (m, 1H, Ar), 7.87–7.88 (m, 1H, Ar), 8.25–8.27 (m, 1H, Ar), 8.74–8.76 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.03 (CH_3), 32.71 (CH_2, Thiaz.), 45.94 (CH_2), 114.90 (d, $^2J_{C\text{-}F}$ = 20.91 Hz, Ar), 115.18 (d, $J_{C\text{-}F}$ = 21.8 Hz, Ar), 120.95 (Pyr), 124.41 (d, $J_{C\text{-}F}$ = 2.6 Hz, Ar), 125.10 (Pyr), 130.90 (d, $J_{C\text{-}F}$ = 8.3 Hz, Ar), 139.24 (d, $J_{C\text{-}F}$ = 7.6 Hz, Ar), 139.24 (Pyr), 149.32 (Pyr), 155.375 (Pyr), 162.56 (d, $J_{C\text{-}F}$ = 244.4 Hz, Ar), 162.77 (C=N, Thiaz.), 163.57 (C=N), 172.80 (C=O). Anal. Calcd. for $C_{17}H_{15}FN_4OS$: C, 59.63; H, 4.42; N, 16.36. Found: C, 59.90; H, 4.67; N, 16.08.

*7.1.17. 3-(4-Fluorobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**17**)*

Yellow powder, mp 78–80 °C, 92% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.94 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 3.98 (s, 2H, CH_2, thiazolidinone), 4.83 (s, 2H, $ArCH_2$), 7.14–7.19 (m, 2H, Ar), 7.37–7.41 (m, 2H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.80 (CH_3), 24.99 (CH_3), 32.35 (CH_2, Thiaz.), 45.46 (CH_2), 115.60 (d, $J_{C\text{-}F}$ = 27.7 Hz, Ar), 130.60 (d, $J_{C\text{-}F}$ = 10.8 Hz, Ar), 132.90 (Ar), 160.15 (d, $J_{C\text{-}F}$ = 44.7 Hz, Ar), 163.59 (C=N, Thiaz.), 165.65 (C=N), 172.58 (C=O). Anal. Calcd. for $C_{13}H_{14}FN_3OS$: C, 55.90; H, 5.05; N, 15.04. Found: C, 55.68; H, 5.24; N, 15.17.

*7.1.18. 3-(4-Fluorobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**18**)*

White powder, mp 79–81 °C, 90% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.71 (bs, 4H, cyclopentane), 2.37–2.39 (m, 4H, cyclopentane), 3.98 (s, 2H, CH_2, thiazolidinone), 4.81 (s, 2H, $ArCH_2$), 7.15–7.19 (m, 2H, Ar), 7.39–7.42 (m, 2H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.60 (CH_2), 24.86 (CH_2), 30.41 (CH_2), 32.39 (CH_2, Thiaz.), 33.06 (CH_2), 45.49 (CH_2), 115.54 (d, $J_{C\text{-}F}$ = 21.5 Hz, Ar), 115.59 (d, $J_{C\text{-}F}$ = 21.5 Hz, Ar), 130.68 (d, $J_{C\text{-}F}$ = 8.4 Hz, Ar), 130.87 (d, $J_{C\text{-}F}$ = 8.4 Hz, Ar), 132.85 (d, $J_{C\text{-}F}$ = 3.1 Hz, Ar), 158.58 (C=N, Thiaz.), 159.69 (C=N), 162.04 (d, $J_{C\text{-}F}$ = 244.8 Hz, Ar), 172.50 (C=O). Anal. Calcd. for $C_{15}H_{16}FN_3OS$: C, 59.00; H, 5.28; N, 13.76. Found: C, 58.81; H, 5.49; N, 13.54.

*7.1.19. 3-(4-Fluorobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**19**)*

Pink powder, mp 137–138 °C, 80% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.47 (s, 3H, CH_3), 3.81 (s, 2H, CH_2, thiazolidinone), 5.00 (s, 2H, $ArCH_2$), 6.93–7.08 (m, 3H, thiophene), 7.22–7.52 (m, 4H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.37 (CH_3), 32.58 (CH_2, Thiaz.), 45.62 (CH_2), 110.00 (Ar), 115.64 (d, $J_{C\text{-}F}$ = 29.3 Hz, Ar), 128.20 (Thioph.), 129.25 (Thioph.), 130.00 (Ar), 130.67 (d, $J_{C\text{-}F}$ = 61.6 Hz, Ar), 132.75 (Thioph.), 143.36 (Ar), 158.18 (C=N, Thiaz.), 162.34 (C=N), 172.67 (C=O). Anal. Calcd. for $C_{16}H_{14}FN_3OS_2$: C, 55.31; H, 4.06; N, 12.09. Found: C, 55.06; H, 3.91; N, 11.84.

*7.1.20. 3-(4-Fluorobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**20**)*

Yellow powder, mp 198–200 °C, 92% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.43 (s, 3H, CH_3), 4.07 (s, 2H, CH_2, thiazolidinone), 4.94 (s, 2H, $ArCH_2$), 7.19 (bs, 2H, Ar), 7.46 (s, 3H, Ar), 7.88 (s, 1H, Ar), 8.06–8.07 (m, 1H, Ar), 8.64–8.66 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.12 (CH_3), 32.66 (CH_2, Thiaz.), 45.77 (CH_2), 115.63 (d, $J_{C\text{-}F}$ = 21.5 Hz, Ar), 115.70 (d, $J_{C\text{-}F}$ = 21.5 Hz, Ar), 120.94 (Pyr), 125.11 (Pyr), 130.76 (d, $J_{C\text{-}F}$ = 8.4 Hz, Ar), 132.75 (d, $J_{C\text{-}F}$ = 3.1 Hz, Ar), 137.10 (Pyr), 149.36 (Pyr), 155.41 (Pyr), 162.09 (d, $J_{C\text{-}F}$ = 244.6 Hz, Ar), 163.55 (C=N, Thiaz.), 164.12 (C=N), 172.78 (C=O, Thiaz). Anal. Calcd. for $C_{17}H_{15}FN_4OS$: C, 59.63; H, 4.42; N, 16.36. Found: C, 59.41; H, 4.24; N, 16.77.

*7.1.21. 3-(2-Chlorobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**21**)*

Yellow oil, 70% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.79 (s, 3H, CH_3), 1.94 (s, 3H, CH_3), 4.05 (s, 2H, CH_2, thiazolidinone), 4.95 (s, 2H, $ArCH_2$), 7.17–7.19 (m, 1H, Ar), 7.28–7.32 (m, 2H, Ar), 7.46–7.47 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.54 (CH_3), 24.93 (CH_3), 32.48 (CH_2, Thiaz.), 43.82 (CH_2), 127.65 (Ar), 128.08 (Ar), 129.70 (Ar), 132.26 (Ar), 133.57 (Ar), 158.39 (Ar), 159.49 (C=N, Thiaz.), 165.70 (C=N), 172.55 (C=N). Anal. Calcd. for $C_{13}H_{14}ClN_3OS$: C, 52.79; H, 4.77; N, 14.21. Found: C, 52.51; H, 4.98; N, 14.03.

*7.1.22. 3-(2-Chlorobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**22**)*

Grey powder, mp 90–92 °C, 93% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.43–1.83 (m, 4H, cyclopentane), 2.34–2.51 (m, 4H, cyclopentane), 3.87 (s, 2H, CH_2, thiazolidinone), 5.15 (s, 2H, $ArCH_2$), 7.10–7.43 (m, 4H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.46 (CH_2), 24.78 (CH_2), 30.17 (CH_2), 32.50 (CH_2, Thiaz.), 32.97 (CH_2), 43.79 (CH_2) 127.67 (Ar), 128.54 (Ar), 129.39 (Ar), 129.71 (Ar), 132.30 (Ar), 133.43 (Ar), 159.23 (C=N, Thiaz.), 172.49 (C=N), 178.32 (C=O). Anal. Calcd. for $C_{15}H_{16}ClN_3OS$: C, 55.98; H, 5.01; N, 13.06. Found: C, 56.14; H, 4.87; N, 12.77.

*7.1.23. 3-(2-Chlorobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**23**)*

Pink powder, mp 100–102 °C, 87% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.31 (s, 3H, CH_3), 3.90 (s, 2H, CH_2, thiazolidinone), 5.18 (s, 2H, $ArCH_2$), 7.05–7.06 (m, 1H, thiophene), 7.22 (bs, 3H, thiophene + Ar), 7.36–7.41 (m, 3H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.12 (CH_3), 32.68 (CH_2, Thiaz.), 43.99 (CH_2), 127.76 (Ar), 128.17 (Ar), 128.25 (Ar), 129.25 (Thioph.), 129.48 (Ar), 129.77 (Thioph.), 132.27 (Thioph.), 133.40 (Ar), 143.29 (Ar), 158.25 (Thioph.), 160.68

(C=N, Thiaz.), 161.99 (C=N), 172.67 (C=O). Anal. Calcd. for $C_{16}H_{14}ClN_3OS_2$: C, 52.81; H, 3.88; N, 11.55. Found: C, 52.66; H, 3.63; N, 11.24.

*7.1.24. 3-(2-Chlorobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**24**)*

Grey powder, mp 149–151 °C, 93% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.42 (s, 3H, CH_3), 3.93 (s, 2H, CH_2, thiazolidinone), 5.22 (s, 2H, $ArCH_2$), 7.24–7.32 (m, 4H, Ar), 7.40–7.41 (m, 1H, Ar), 7.73 (bs, 1H, Ar), 8.18–8.19 (m, 1H, Ar), 8.63 (bs, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.91 (CH_3), 32.77 (CH_2, Thiaz.), 44.13 (CH_2), 120.92 (Pyr), 125.10 (Pyr), 127.79 (Ar), 128.57 (Ar), 129.53 (Ar), 129.83 (Ar), 132.33 (Ar), 133.37 (Ar), 137.07 (Pyr), 149.32 (Pyr), 155.34 (Pyr), 163.63 (C=N, Thiaz.), 163.70 (C=N), 172.74 (C=O). Anal. Calcd. for $C_{17}H_{15}ClN_4OS$: C, 56.90; H, 4.21; N, 15.61. Found: C, 56.71; H, 4.39; N, 15.78.

*7.1.25. 3-(3-Chlorobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**25**)*

White powder, mp 90–92 °C, 97% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.13 (s, 6H. 2 × CH_3), 3.84 (s, 2H, CH_2, thiazolidinone), 4.96 (s, 2H, $ArCH_2$), 7.14–7.54 (m, 4H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.74 (CH_3), 25.00 (CH_3), 32.41 (CH_2, Thiaz.), 45.66 (CH_2), 127.06 (Ar), 127.99 (Ar), 128.34 (Ar), 130.76 (Ar), 133.40 (Ar), 139.06 (Ar), 159.82 (C=N, Thiaz.), 165.70 (C=N), 172.61 (C=N). Anal. Calcd. for $C_{13}H_{14}ClN_3OS$: C, 52.79; H, 4.77; N, 14.21. Found: C, 52.94; H, 4.53; N, 14.50.

*7.1.26. 3-(3-Chlorobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**26**)*

White powder, mp 121–123 °C, 90% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.74–1.75 (m, 4H, cyclopentane), 2.35–2.41 (m, 4H, cyclopentane), 4.04 (s, 2H, CH_2, thiazolidinone), 4.87 (s, 2H, $ArCH_2$), 7.35–7.45 (m, 4H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.57 (CH_2), 24.86 (CH_2), 30.41 (CH_2), 32.44 (CH_2, Thiaz.), 33.03 (CH_2), 45.66 (CH_2) 127.30 (Ar), 128.03 (Ar), 128.61 (Ar), 130.76 (Ar), 133.34 (Ar), 138.96 (Ar), 159.56 (C=N, Thiaz.), 172.53 (C=O). Anal. Calcd. for $C_{15}H_{16}ClN_3OS$: C, 55.98; H, 5.01; N, 13.06. Found: C, 55.67; H, 5.19; N, 13.24.

*7.1.27. 3-(3-Chlorobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**27**)*

Pink powder, mp 129–131 °C, 89% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.26 (s, 3H, CH_3), 3.62 (s, 2H, CH_2, thiazolidinone), 4.78 (s, 2H, $ArCH_2$), 6.85–6.88 (m, 1H, thiophene), 7.05–7.08 (m, 3H, thiophene + Ar), 7.17–7.20 (m, 2H, Ar), 7.31 (s, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.34 (CH_3), 32.66 (CH_2, Thiaz.), 45.81 (CH_2), 127.13 (Ar), 128.06 (Ar), 128.21 (Thioph.), 128.44 (Ar), 129.29 (Thioph.), 130.03 (Thioph.), 130.80 (Ar), 133.44 (Ar), 138.95 (Ar), 143.38 (Thioph.), 158.26 (C=N, Thiaz.), 162.24 (C=N), 172.71 (C=O). Anal. Calcd. for $C_{16}H_{14}ClN_3OS_2$: C, 52.81; H, 3.88; N, 11.55. Found: C, 52.99; H, 3.69; N, 11.73.

*7.1.28. 3-(3-Chlorobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**28**)*

Grey powder, mp 147–149 °C, 83% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.59 (s. 3H, CH_3), 3.86 (s, 2H, CH_2, thiazolidinone), 5.03 (s, 2H, $ArCH_2$), 7.25–7.41 (m, 4H, Ar), 7.48–7.56 (m, 1H, Ar), 7.70–7.76 (m, 1H, Ar), 8.15–8.21 (m, 1H, Ar), 8.61–8.66 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.06 (CH_3), 32.72 (CH_2, Thiaz.), 45.91 (CH_2), 120.95 (Pyr), 125.11 (Pyr), 127.19 (Ar), 128.10 (Ar), 128.46 (Ar), 130.82 (Ar), 133.46 (Ar), 137.08 (Pyr), 138.87 (Ar), 149.34 (Pyr), 155.37 (Pyr), 163.60 (C=N, Thiaz.), 164.02 (C=N), 172.80 (C=O). Anal. Calcd. for $C_{17}H_{15}ClN_4OS$: C, 56.90; H, 4.21; N, 15.61. Found: C, 56.65; H, 4.07; N, 15.37.

*7.1.29. 3-(4-Chlorobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**29**)*

White powder, mp 103–105 °C, 90% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.66 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 3.81 (s, 2H, CH_2, thiazolidinone), 4.95 (s, 2H, $ArCH_2$), 7.22–7.47 (m, 4H, Ar). ^{13}C NMR (101 MHz, $CDCl_3$) δ 18.83 (CH_3), 25.04 (CH_3), 32.35 (CH_2, Thiaz.), 45.88 (CH_2), 128.57 (Ar), 130.35 (Ar), 134.27 (Ar), 145.24 (Ar), 159.08 (C=N, Thiaz.), 166.48 (C=N), 171.89 (C=O). Anal. Calcd. for $C_{13}H_{14}ClN_3OS$: C, 52.79; H, 4.77; N, 14.21. Found: C, 52.46; H, 4.59; N, 14.10.

*7.1.30. 3-(4-Chlorobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**30**)*

Grey powder, mp 79–81 °C, 96% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.71 (m, 4H, cyclopentane), 2.37 (m, 4H, cyclopentane), 3.99 (s, 2H, CH_2, thiazolidinone), 5.21 (s, 2H, $ArCH_2$), 7.35–7.40 (m, 4H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.58 (CH_2), 24.84 (CH_2), 30.39 (CH_2), 32.39 (CH_2, Thiaz.), 33.03 (CH_2), 45.51 (CH_2), 128.78 (Ar), 130.54 (Ar), 132.53 (Ar), 135.55 (Ar), 159.64 (C=N, Thiaz.), 172.49 (C=N), 178.19 (C=O). Anal. Calcd. for $C_{15}H_{16}ClN_3OS$: C, 55.98; H, 5.01; N, 13.06. Found: C, 56.16; H, 4.82; N, 12.84.

*7.1.31. 3-(4-Chlorobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**31**)*

White powder, mp 149–151 °C, 81% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.34 (s, 3H, CH_3), 4.03 (s, 2H, CH_2, thiazolidinone), 4.90 (s, 2H, $ArCH_2$), 7.12 (bs, 1H, thiophene), 7.41 (bs, 4H, Ar), 7.54 (bs, 1H, thiophene), 7.61–7.62 (m, 1H, thiophene). ^{13}C NMR (101 MHz, slightly soluble in DMSO-d_6) δ 15.37 (CH_3), 32.61 (CH_2, Thiaz.), 45.66 (CH_2), 128.22 (Ar), 128.86 (Thioph.), 129.28 (Ar), 130.03 (Thioph.), 130.35 (Thioph.), 130.80 (Ar), 132.67 (Ar), 135.48 (Ar), 143.36 (Thioph.), 158.24 (C=N, Thiaz.), 162.28 (C=N), 172.70 (C=O). Anal. Calcd. for $C_{16}H_{14}ClN_3OS_2$: C, 52.81; H, 3.88; N, 11.55. Found: C, 53.05; H, 4.02; N, 11.32.

*7.1.32. 3-(4-Chlorobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**32**)*

Grey powder, mp 202–204 °C, 94% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.41 (s, 3H, CH_3), 4.08 (s, 2H, CH_2, thiazolidinone), 4.94 (s, 2H, $ArCH_2$), 7.43 (s, 5H, Ar), 7.86–7.88 (m, 1H, Ar), 8.05–8.07 (m, 1H, Ar), 8.64 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.12 (CH_3), 32.68 (CH_2, Thiaz.), 45.81 (CH_2), 120.94 (Pyr), 125.12 (Pyr), 128.90 (Ar), 130.42 (Ar), 132.74 (Ar), 135.50 (Ar), 137.11 (Pyr), 149.36 (Pyr), 155.39 (Pyr), 163.59 (C=N, Thiaz.), 164.07 (C=N), 172.78 (C=O). Anal. Calcd. for $C_{17}H_{15}ClN_4OS$: C, 56.90; H, 4.21; N, 15.61. Found: C, 57.11; H, 3.98; N, 15.94.

*7.1.33. 3-(4-Aminobenzyl)-2-(2-(propan-2-ylidene)hydrazono) thiazolidin-4-one (**33**)*

Yellow powder, mp 166–168 °C, 69% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.97 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 3.92 (s, 2H, CH_2, thiazolidinone), 4.65 (s, 2H, $ArCH_2$), 5.06 (bs, 2H, NH_2, D_2O exch.), 6.46–6.48 (d, J = 8.4 Hz, 2H, Ar), 7.03–7.05 (d, J = 8.4 Hz, 2H, Ar). ^{13}C NMR (101 MHz, $CDCl_3$) δ 18.11 (CH_3), 25.43 (CH_3), 32.78 (CH_2, Thiaz.), 45.65 (CH_2), 125.12 (Ar), 130.88 (Ar), 132.98 (Ar), 134.64 (Ar), 160.68 (C=N, Thiaz.), 166.32 (C=N), 171.12 (C=O). Anal. Calcd. for $C_{13}H_{16}N_4OS$: C, 56.50; H, 5.84; N, 20.27. Found: C, 56.35; H, 5.61; N, 20.04.

*7.1.34. 3-(4-Aminobenzyl)-2-(2-cyclopentylidenehydrazono) thiazolidin-4-one (**34**)*

Yellow powder, mp 208–210 °C, 71% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.79–1.87 (m, 4H, cyclopentane), 2.47–2.51 (t, 2H, cyclopentane), 2.53–2.59 (t, 2H, cyclopentane), 3.66 (bs, 2H, NH_2,

D_2O exch.), 3.74 (s, 2H, CH_2, thiazolidinone), 4.84 (s, 2H, $ArCH_2$), 6.61–6.63 (d, J = 8.4 Hz, 2H, Ar), 7.30–7.32 (d, J = 8.4 Hz, 2H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.61 (CH_2), 24.89 (CH_2), 30.50 (CH_2), 32.30 (CH_2, Thiaz.), 33.03 (CH_2), 46.03 (CH_2), 113.80 (Ar), 123.49 (Ar), 130.11 (Ar), 148.72 (C=N, Thiaz.), 159.92 (C=N), 172.44 (Ar), 177.97 (C=O). Anal. Calcd. for $C_{15}H_{18}N_4OS$: C, 59.58; H, 6.00; N, 18.53. Found: C, 59.31; H, 5.74; N, 18.32.

*7.1.35. 3-(4-Aminobenzyl)-2-(2-(1-(thiophen-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**35**)*

Yellow powder, mp 163–165 °C, 70% yield; ^1H NMR (400 MHz, DMSO-d_6): δ 2.42 (s, 3H, CH_3), 3.97 (s, 2H, CH_2, thiazolidinone), 4.72 (s, 2H, $ArCH_2$), 5.06 (bs, 2H, NH_2, D_2O exch.), 6.49–6.51 (d, J = 8.0 Hz, 2H, Ar), 7.07–7.09 (d, J = 8.0 Hz, 2H, Ar), 7.12 (bs, 1H, thiophene), 7.55 (bs, 1H, thiophene), 7.61–7.62 (m, 1H, thiophene). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.45 (CH_3), 32.51 (CH_2, Thiaz.), 46.26 (CH_2), 113.93 (Ar), 123.47 (Ar), 128.20 (Thioph.), 129.14 (Thioph.), 129.93 (Ar), 143.53 (Thioph.), 148.78 (Ar), 157.93 (C=N, Thiaz.), 162.61 (C=N), 172.66 (C=O). Anal. Calcd. for $C_{16}H_{16}N_4OS_2$: C, 55.79; H, 4.68; N, 16.27. Found: C, 55.61; H, 4.37; N, 16.45.

*7.1.36. 3-(4-Aminobenzyl)-2-(2-(1-(pyridin-2-yl)ethylidene) hydrazono)thiazolidin-4-one (**36**)*

Green powder, mp 197–198 °C, 72% yield; ^1H NMR (400 MHz, $CDCl_3$): δ 2.62 (s, 3H, CH_3), 3.71 (bs, 2H, NH_2, D_2O exch.), 3.79 (s, 2H, CH_2, thiazolidinone), 4.95 (s, 2H, $ArCH_2$), 6.64–6.66 (d, J = 8.0 Hz, 2H, Ar), 7.39–7.40 (m, 3H, Ar), 7.72–7.75 (m, 1H, Ar), 8.20–8.22 (d, J = 7.6 Hz, 1H, Ar), 8.65–8.66 (m, 1H, Ar). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.16 (CH_3), 32.54 (CH_2, Thiaz.), 46.34 (CH_2), 113.89 (Pyr), 120.89 (Pyr), 123.33 (Ar), 125.06 (Ar), 130.00 (Ar), 137.08 (Ar), 148.81 (Pyr), 149.33 (Pyr), 155.43 (Pyr), 163.23 (C=N, Thiaz.), 164.43 (C=N), 172.73 (C=O). Anal. Calcd. for $C_{17}H_{17}N_5OS$: C, 60.16; H, 5.05; N, 20.63. Found: C, 60.38; H, 5.26; N, 20.80.

7.2. Antibacterial activity

Microtiter plates containing serial dilutions of each compound ranging from 256 to 0.5 μg/mL were inoculated with each organism to yield the appropriate density (10^5/mL) in a 100 μL final volume; each plate included positive controls (bacteria without the compound), and a negative control (medium only). The plates were incubated for 18–22 h at 35 °C. The minimum inhibitory concentration (MIC) for all isolates was defined as the lowest concentration of antibacterial agent that completely inhibited the growth of the organism, as detected by the unaided eye.

7.3. Antifungal activity

Microtiter plates containing serial dilutions of each compound were inoculated with each organism to yield the appropriate density (10^3/mL) in a 100 μL final volume; each plate included positive controls (fungi without a compound) and a negative control (medium only). The plates were incubated for 24 h at 37 °C. The MIC for all isolates was defined as the lowest concentration of antifungal agents that completely inhibited the growth of the organism, as detected by unaided eye.

7.4. Cytotoxicity assay

The Hep2 cell line, delivered from a human epidermoid carcinoma of the larynx and purchased from a Korean Cell Line Bank (KCLB No. 10023), was a kind gift by Prof. R. Misasi (Department of Experimental Medicine-Sapienza University of Rome). Cells were cultured in Dulbecco's modified eagle media (DMEM), supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% streptomycin-penicillin. Cell lines were maintained as adherent type cultures under humidified atmosphere in 5% CO_2 at 37 °C in Dulbecco's modified Eagle's culture medium. Experiments were performed on cells grown to 60–70% confluency. The stock solutions of the investigated compounds were prepared in sterile DMSO and successive dilutions were made in culture medium; the percentage of DMSO present in culture medium never exceeded 0.5%. Hep2 cells in the exponential phase of growth (1×10^5/mL) were seeded into 24-well microplate and incubated for 24 h with five different concentrations of the compounds (0.05–100 μg/mL). Some plates containing cells alone or cells and DMSO represented the negative controls, whereas cells incubated with 1 mM natrium nitroprusside represented the positive one. After incubation time, cells were mechanically scraped off from the plates, resuspended in fresh medium and incubated for 30 min with gentle shaking at 37 °C in atmosphere of 5% CO_2 to recover from the eventual stress. An aliquot was then diluted (1:1) with a solution 0.4% Trypan blue stain. After few minutes at room temperature, cells were counted under an optical microscope in a Thoma hemocytometer chamber by two different operators. On the basis that Trypan blue is a vital dye and can enter and interact with the cells unless the plasmatic membrane is damaged, blue stained cells were considered as having died. Values are expressed as % of viable cells. Cell viability in control samples was always 97–98%.

7.5. Radical scavenging activity: free radical scavenging activity (DPPH) and ABTS radical cation scavenging ability

The effects of the samples on DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2 azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] cation radicals were estimated according to well established procedure in the literature [31,32].

DPPH assay: sample solution (1 mL) was added to a 1 mL methanol solution of DPPH (final concentration of DPPH was 0.2 mM). The sample absorbance was read at 517 nm after incubation for 30 min at room temperature in the dark. The corresponding IC_{50} value, which is the effective concentration at which 50% of DPPH radicals are scavenged, was calculated for each compounds and trolox (reference standard).

ABTS assay: briefly, a $ABTS^{\bullet+}$ radical cation was produced directly by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 12–16 h in the dark at the room temperature. Prior to beginning the assay, ABTS solution was diluted with methanol to obtain an absorbance of 0.700 ± 0.02 at 734 nm. Then, sample solution (1 mL) was added to the ABTS solution (2 mL) and mixed. The sample absorbance was read at 734 nm after incubation for 30 min at room temperature [32]. Trolox is used as a positive control and the IC_{50} of each compound was then calculated.

7.6. Metal chelating activity on ferrous ions

Metal chelating activity on ferrous ions was evaluated by the method described by Aktumsek et al. [33]. Briefly, the sample solution (2 mL) was added to $FeCl_2$ solution (0.05 mL, 2 mM). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Similarly, a blank was prepared by adding sample solution (2 mL) to $FeCl_2$ solution (0.05 mL, 2 mM) and water (0.2 mL) without ferrozine. Then, the sample and blank absorbance were read at 562 nm after incubation for 10 min at room temperature. The absorbance of blank was subtracted from that of the sample. EDTA is used as a positive control and this biological property was expressed as IC_{50} value for each compound.

7.7. Reducing power: cupric ion reducing (CUPRAC) and ferric reducing antioxidant power (FRAP) methods

Cupric ion reducing (CUPRAC) method: sample solution (0.5 mL) was added to the premixed reaction mixture containing $CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding the sample solution (0.5 mL) to the premixed reaction mixture (3 mL) without $CuCl_2$. Then, the sample and blank absorbance were read at 450 nm after incubation for 30 min at room temperature [34]. The EC_{50}, which is the effective concentration at which the absorbance was 0.5, was calculated for each compound and trolox (reference standard).

7.8. Ferric reducing antioxidant power (FRAP) method

sample solution (0.1 mL) was added to the premixed FRAP reagent (2 mL) containing acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1 (v:v:v). Then, the sample absorbance was read at 593 nm after incubation for 30 min at room temperature [33]. The results were evaluated by using EC_{50} values.

7.9. Total antioxidant activity by phosphomolybdenum method

Total antioxidant activities were evaluated using the phosphomolybdenum method as previously described in the literature [35]. Sample solution (0.2 mL) was combined with 2 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample absorbance was read at 695 nm after incubation for 90 min at 95 °C. The results were expressed as EC_{50} values.

Conflict of interest

None declared.

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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.2016.04.012.

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