Discovery of novel 1,2,4-triazolo-1,2,4-triazines with thiomethylpyridine hinge binders as potent c-Met kinase inhibitors

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Discovery of novel 1,2,4-triazolo-1,2,4-triazines with thiomethylpyridine hinge binders as potent c-Met kinase inhibitors

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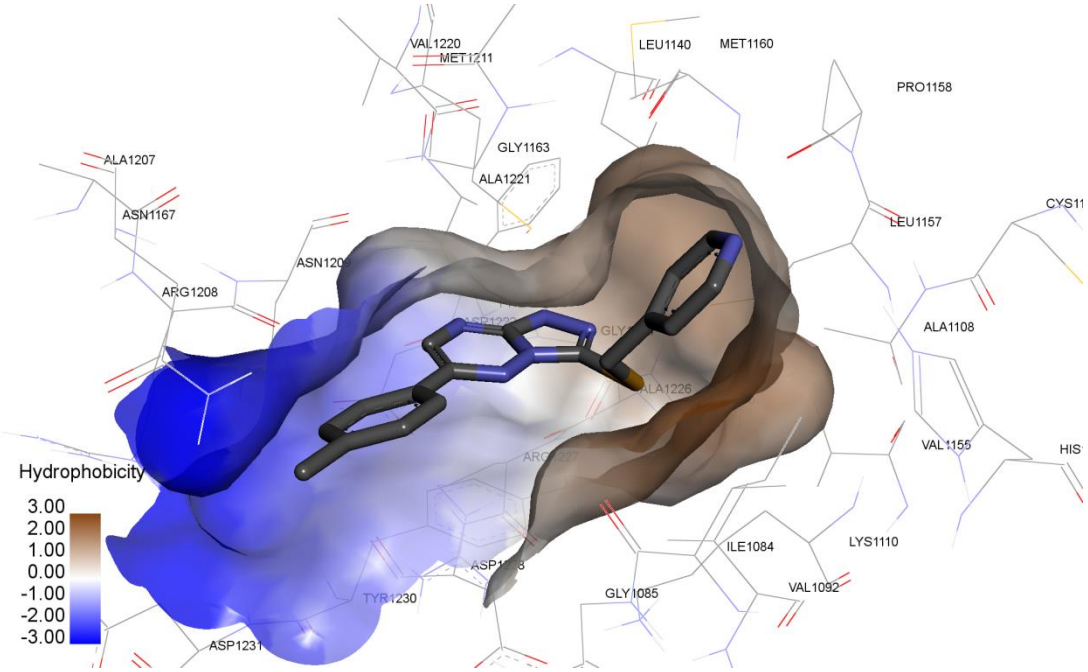
Running title: Triazolotriazines as c-Met kinase inhibitors

Graphical Abstract



Compound ***10b*** was introduced as a potent c-Met kinase inhibitor with IC_{50} of 4.6 nM and was more cytotoxic than crizotinib on HepG2 cells.

Aim: Mesenchymal-epithelial transition factor HGF/c-Met overactivation is involved in diverse human cancers. Herein, we report the synthesis and biological evaluation of thiomethylpyridine-linked triazolotriazines as c-Met kinase inhibitors.

Results: Compounds ***10b*** and ***11e*** were more potent than crizotinib on HepG2 cells with IC_{50} values of 0.74 and 0.71 μM in the MTT assay, respectively. Interestingly, all of the target compounds displayed IC50 values in the range of 3.9-11.1 nM in the c-Met kinase inhibition assay which were lower than the value for crizotinib (11.1 nM).

Conclusion: Target compound ***10b*** can be considered as a leading drug candidate due to its lower IC50 values than crizotinib in both HGF-induced proliferation and c-Met kinase inhibition assays.

Keywords: Synthesis, [1,2,4]triazolo[4,3-b][1,2,4]triazine, Anticancer, c-Met kinase, Caspase 3/7, X-ray crystallography

Abstract

Mesenchymal-epithelial transition factor (c-Met) pertaining to receptor tyrosine kinases is a cell-surface receptor for hepatocyte growth factor (HGF). c-Met is widely distributed in the brain, human liver, gastrointestinal tract, and kidney. c-Met signaling leads to cell proliferation, migration, survival and embryonic morphogenesis [1]. HGF/c-Met over-activation is involved in diverse human tumors, such as glioblastomas, various carcinomas in breast, lung, liver and prostate, and in ovarian cancer [2]. Different strategies have been employed to regulate HGF/c-Met signaling pathway using HGF antagonists, c-Met monoclonal antibodies, anti-HGF antibodies and small-molecule c-Met inhibitors [3]. Small-molecule c-Met inhibitors, on the basis of their binding modes, have been subdivided into three types, type I, II and III. Type I inhibitors occupy the ATP binding pocket in the form of a "U" conformation and display high selectivity on the c-Met enzyme. Type II inhibitors have extended conformation in the ATP binding pocket of c-Met and do not have selectivity over the c-MET enzyme [4, 5]. SGX-523 (Figure 1), a type I and selective c-MET inhibitor has showed excellent antitumor activity on the U87MG xenograft model [6]. Based on previous reports, triazolotriazines bearing 4-hydroxyphenylmethyl at the 3-position and O-linked triazolotriazines demonstrated superior c-met inhibitory activity (Figure 1, compounds 1 and 2) [7]. In the same compounds, the presence of a hydrogen-bond acceptor atom like nitrogen in the quinoline motif (Figure 1) was proved to constitute a crucial hydrogen bond with NH atom of Met1160 in the hinge binding region of the c-Met active site [8]. With respect to the importance of the electron-deficient triazolotriazine core in the formation of a strong π-π stacking interaction with Tyr-1230 in the c-Met activation loop, which confers potency and selectivity for c-Met inhibition against a wide range of

kinases, we designed and synthesized the 6-aryl-triazolotriazines, ***10a-f*** and ***11a-f*** (Figure 1), linked through thiomethylene to a 3- or 4-pyridyl ring. The flexible thiomethylene side chain containing the pyridine motif as a hinge binder ensures the formation of an essential hydrogen bond with Met1160. The effect of introducing the new thiomethylene linker to attach the hinge binder pyridine ring to the triazolotriazine core has been investigated for c-Met kinase inhibition and cell proliferation assays.

The chemical structure of synthesized titled compounds was confirmed by X-ray diffraction, ^{1}H and ^{13}C-NMR, as well as Mass spectrometry. The anticancer activity of the target compounds was evaluated by HGF-induced proliferation assay against A549, HepG2, MCF7 and MDA-MB231 cell lines and compared to crizotinib as a selective c-MET inhibitor. The extent of cytotoxicity and apoptosis mediated by Caspase 3/7 activity were assessed and finally, the ability of the target compounds to inhibit c-Met kinase was evaluated and expressed as enzyme percent inhibition.

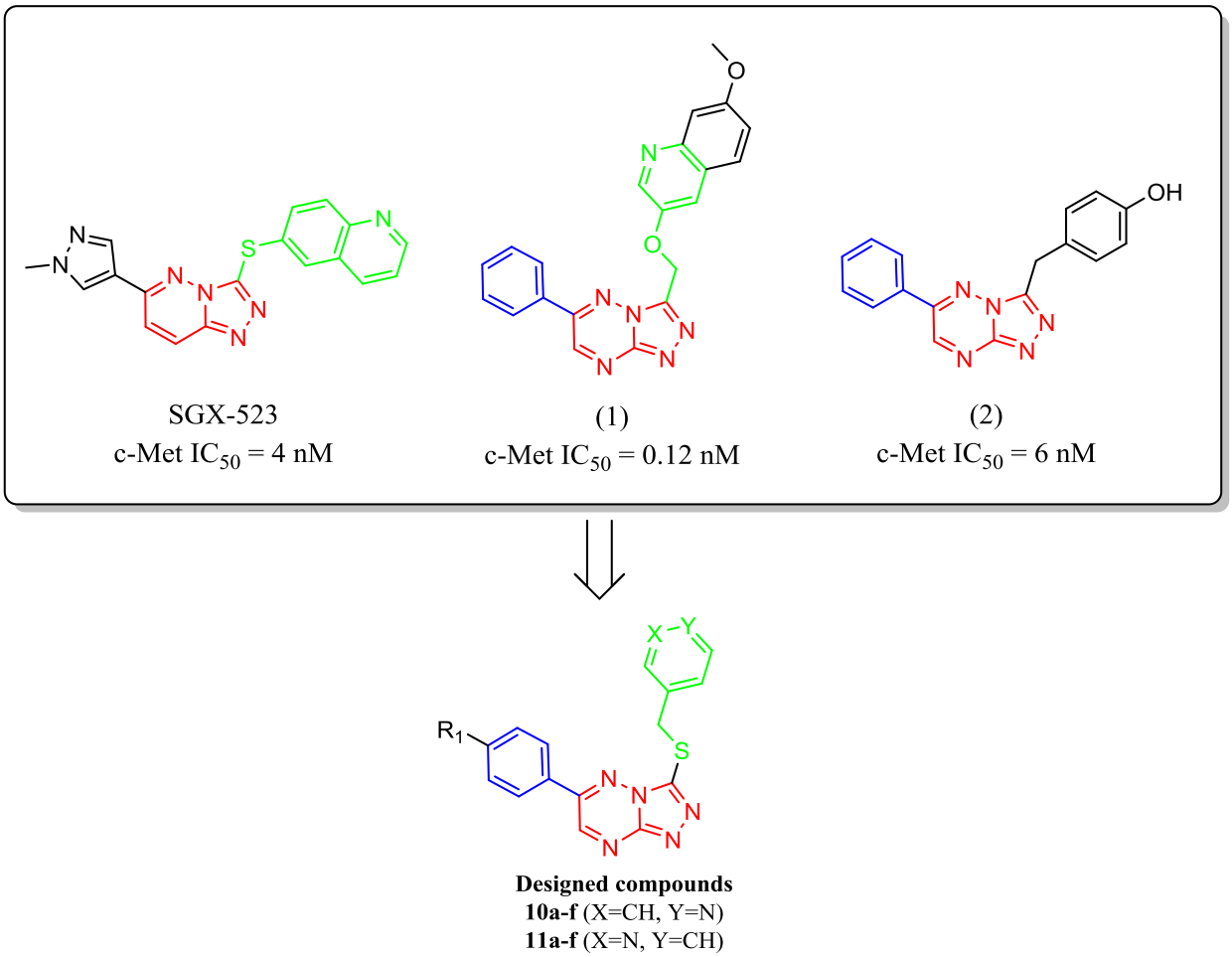


Figure 1. Schematic representation of designed triazolotriazines ***10a-f*** and ***11a-f***, based on the previous c-Met inhibitors.

Experimental section

Material & Methods

All chemical reagents and solvents were purchased from Sigma–Aldrich or Merck suppliers and used without further purification. Crizotinib and HGF were purchased from Sigma. The progress of reactions was checked by Thin-layer chromatography (TLC) using TLC Silica gel 60 F_{254} (Merck, Darmstadt, Germany). A UV cabinet with wavelengths 254 and 360 nm was used for visualizing spots on the TLC plates. Column chromatography was performed using Silica gel 60 (0.040-0.063 mm), Merck, Germany. Melting points were measured in open capillaries on a Stuart Scientific apparatus and are uncorrected. IR spectra were recorded on a FT-IR Perkin

Elmer spectrophotometer. NMR spectra were recorded on Bruker 500 MHz spectrometer and tetramethylsilane (TMS) was used as the standard and chemical shifts are expressed in ppm units. Coupling constants are reported in Hertz (Hz). Low resolution mass spectra were measured using a HP 5975 Mass Selective Detector (Agilent technologies). Elemental analyses for C, H and N were performed by using a CHN elemental analyzer (GmbH-Germany), and the results are within ±0.4% of the theoretical values.

Preparation of methyl hydrazinecarbimidothioate hydroiodide.

A mixture of thiosemicarbazide (1 mmol) and iodomethane (10 mmol) in absolute ethanol were heated at 50°C for 3h. After cooling to r.t. a white precipitate was filtrated and air dried. Yield: 64%. m.p. 138°C.

*General procedure for the preparation of 2,2-dihydroxy-1-arylethanones **4a-f***

To a suspension of SeO_2 (1 equiv.) in a mixture of 1,4-dioxane and H_2O (21:1) was added acetophenones **3a-f** (1 equiv.) which was then heated at reflux for 2h. The reaction mixture was cooled to room temperature and filtered through Celite. The filtrate was concentrated in vacuo, water was added and then heated at reflux for 5h. Next, the reaction mixture was cooled in an ice bath to begin precipitation. The white precipitate obtained was filtered and dried in an oven.

*2,2-Dihydroxy-1-phenylethanone (**4a**).* Yield: 57%. White powder. m.p. 83-85°C. IR (KBr, cm^{-1}): 3405 (O-H), 1697 (C=O).

*2,2-Dihydroxy-1-(p-tolyl)ethanone (**4b**).* Yield: 55%. White powder. m.p. 113-115°C. IR (KBr, cm^{-1}): 3399 (O-H), 2915 (aliphatic C-H), 1687 (C=O). ^1H NMR (500 MHz, $CDCl_3$) δ: 2.47 (s, 3H), 7.34 (d, J = 8.2 Hz, 2H), 8.13 (d, J = 8.3 Hz, 2H), 9.68 (s, 1H).

*2,2-Dihydroxy-1-(4-methoxyphenyl)ethanone (**4c**).* Yield: 56%. White powder. m.p. 111-114°C. IR (KBr, cm^{-1}): 3419 (O-H), 2987, 2944, 2846 (aliphatic C-H), 1682 (C=O).

*2,2-Dihydroxy-1-(4-hydroxyphenyl)ethanone (**4d**).* Yield: 50%. White powder. m.p. 114-116°C.

*1-(4-Bromophenyl)-2,2-dihydroxyethanone (**4e**).* Yield: 41%. White powder. m.p. 131-133°C. IR (KBr, cm^{-1}): 3378 (O-H), 2974 (aliphatic C-H), 1695 (C=O). ^1H NMR (500 MHz, $CDCl_3$) δ: 3.99 (s, 2H), 5.93 (s, 1H), 7.70 (d, *J* = 8.3 Hz, 2H), 8.11 (d, *J* = 8.4 Hz, 2H), 9.65 (s, 1H).

*1-(4-Chlorophenyl)-2,2-dihydroxyethanone (**4f**).* Yield: 35%. White powder. m.p. 96-98°C. IR (KBr, cm^{-1}): 3374 (O-H), 2978 (aliphatic C-H), 1697 (C=O). ^1H NMR (500 MHz, $CDCl_3$) δ: 7.51 (d, *J* = 8.3 Hz, 2H), 8.18 (d, *J* = 7.7 Hz, 2H), 9.64 (s, 1H).

*General procedure for the preparation of 2,2-diethoxy-1-arylethanones **5a-f***

To a solution of 2,2-dihydroxy-1-arylethanones, ***4a-f*** (1 equiv.) in dichloromethane, *p*-toluenesulfonic acid (PTSA, 0.05 equiv.) and triethyl orthoformate (2 equiv.) were added and heated at reflux for 2h. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel (230–400 mesh) using chloroform as eluent.

*2,2-Diethoxy-1-phenylethanone (**5a**).* Yield: 90%. Yellow oil. IR (KBr, cm^{-1}): 3068 (aromatic C-H), 2979, 2932, 2882 (aliphatic C-H), 1689 (C=O). ^1H NMR (500 MHz, $CDCl_3$) δ: 1.26 (t, *J* = 7.0 Hz, 6H), 3.64-3.70 (m, 2H), 3.75-3.81 (m, 2H), 5.30 (s, 1H), 7.45-7.49 (m, 2H), 7.75-7.60 (m, 1H), 8.17-8.19 (m, 2H).

*2,2-Diethoxy-1-(p-tolyl)ethanone (**5b**).* Yield: 80%. Yellow oil. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.26 (t, *J* = 7.0 Hz, 6H), 2.42 (s, 3H), 3.63-3.70 (m, 2H), 3.73-3.80 (m, 2H), 5.29 (s, 1H), 7.27 (d, *J* = 8.1 Hz, 2H), 8.08 (d, *J* = 8.2 Hz, 2H).

*2,2-Diethoxy-1-(4-methoxyphenyl)ethanone (**5c**).* Yield: 91%. Yellow oil. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.25 (t, *J* = 7.0, 6H), 3.62-3.68 (m, 2H), 3.73-3.80 (m, 2H), 3.88 (s, 3H), 5.25 (s, 1H), 6.94 (d, *J* = 9 Hz, 2H), 8.17 (d, *J* = 8.9 Hz, 2H).

*2,2-Diethoxy-1-(4-hydroxyphenyl)ethanone (**5d**).* Yield: 90%. Pale yellow oil. The title compound ***5d*** was purified by column chromatography, eluting with 5% ethylacetate in chloroform. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.25 (t, *J* = 7.0 Hz, 6H), 3.64-3.70 (m, 2H), 3.72-3.78 (m, 2H), 5.33 (s, 1H), 6.93 (d, *J* = 8.8 Hz, 2H), 8.12 (d, *J* = 8.8 Hz, 2H).

*1-(4-Bromophenyl)-2,2-diethoxyethanone (**5e**).* Yield: 88%. Pale yellow oil. IR (KBr, cm^{-1}): 2978, 2931, 2882 (aliphatic C-H), 1693 (C=O). ^1H NMR (500 MHz, $CDCl_3$) δ: 1.10 (t, *J* = 6.9

Hz, 6H), 3.47-3.52 (m, 2H), 3.61-3.65 (m, 2H), 5.04 (s, 1H), 7.43 (d, *J* = 7.9 Hz, 2H) , 7.92 (d, *J* = 7.2 Hz, 2H).

*1-(4-Chlorophenyl)-2,2-diethoxyethanone (**5f**).* Yield: 85%. Pale yellow oil. . IR (KBr, cm^{-1}): 3441 (aromatic C-H), 2979, 2931, 2896 (aliphatic C-H), 1693 (C=O).

*General procedure for the preparation of 3-(methylthio)-6-aryl-1,2,4-triazines **6a-f.***

To a solution of *2,2-diethoxy-1-arylethanones, **5a-c*** and ***5e-f*** (1 equiv.) in absolute ethanol were added PTSA (0.05 equiv.) and thiosemicarbazide (1 equiv.) and stirred at r.t. for 8h. The completion of the reaction was monitored by TLC. After completion, 1.1 equiv. of methyl iodide was added to the mixture and stirring was continued overnight. The solvent was removed under reduced pressure and acetic acid was added to the obtained residue and heated at 60 °C overnight with stirring. Finally, the resulting solution was concentrated *in vacuo* and the crude product was purified by column chromatography (silica gel, 230–400 mesh) eluting with chloroform.

*3-(Methylthio)-6-phenyl-1,2,4-triazine (**6a**).* Yield: 50%. Brown powder. m.p. 93-95°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.82 (s, 3H), 7.61-7.65 (m, 3H), 8.12-8.14 (m, 2H), 8.87 (s, 1H); MS: (m/z, %): 203.1 (M^+, 30), 175.1 (10), 102.2 (100), 76.1 (25).

*3-(Methylthio)-6-(p-tolyl)-1,2,4-triazine (**6b**).* Yield: 30%. Brown powder. m.p. 119-120°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.46 (s, 3H), 2.74 (s, 3H), 7.37 (d, *J* = 8 Hz, 2H), 7.96 (d, *J* = 8.2 Hz, 2H), 8.77 (s, 1H); MS: (m/z, %): 217.2 (M^+, 31), 116.2 (100), 89.1 (14).

*6-(4-Methoxyphenyl)-3-(methylthio)-1,2,4-triazine (**6c**).* Yield: 40%. Brown powder. m.p. 140-143°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.74 (s, 3H), 3.90 (s, 3H), 7.07 (d, *J* = 8.9 Hz, 2H), 8.02 (d, *J* = 8.8 Hz, 2H), 8.75 (s, 1H); MS: (m/z, %): 233.1 (M^+, 82), 132.2 (100), 117.1 (74), 89.1 (66).

*4-(3-(Methylthio)-1,2,4-triazin-6-yl)phenol (**6d**).* Yield: 41%. To a suspension of *2,2-diethoxy-1-(4-hydroxyphenyl)ethanone, **5d*** (1 equiv.) in absolute ethanol, PTSA (0.05 equiv.) and methyl hydrazinecarbimidothioate hydroiodide (1 equiv.) were added and stirred at r.t. for 8h. The solution was concentrated under vacuum, then acetic acid was added and heated at 60 °C overnight with stirring. The crude product was purified on a silica gel column, eluting with 5% ethylacetate in chloroform to give the yellow powder. m.p. 147-150°C. ^{1}H NMR (500 MHz,

DMSO-d_6) δ: 2.65 (s, 3H), 6.94 (d, *J* = 8.7 Hz, 2H), 8.01 (d, *J* = 8.7 Hz, 2H), 9.13 (s, 1H), 10.07 (s, 1H); MS: (m/z, %): 219.1 (M^+, 86), 118.2 (100), 89.1 (65).

*6-(4-Bromophenyl)-3-(methylthio)-1,2,4-triazine (**6e**).* Yield: 54%. The residue was crystallized from ethanol to afford ***6e*** as brown crystals. m.p. 157-159°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.74 (s, 3H), 7.70 (d, *J* = 8.5 Hz, 2H), 7.94 (d, *J* = 8.5 Hz, 2H), 8.77 (s, 1H); MS: (m/z, %): 281.0 (M^+, 19), 283.1 (M+2, 19), 180.1 (100), 101.1 (57).

*6-(4-Chlorophenyl)-3-(methylthio)-1,2,4-triazine (**6f**).* Yield: 37%. Brown powder. m.p. 155-157°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.74 (s, 3H), 7.53 (d, *J* = 8.6 Hz, 2H), 8.01 (d, *J* = 8.6 Hz, 2H), 8.77 (s, 1H); MS: (m/z, %): 237.1 (M^+, 20), 239.1 (M+2, 7.7), 136.2 (100), 101.1 (29).

*General procedure for the preparation of 3-(methylsulfonyl)-6-aryl-1,2,4-triazines **7a-f***

To a suspension of *3-(methylthio)-6-aryl-1,2,4-triazines, **6a-f*** (1 equiv.) in THF was added potassium peroxymonosulfate (oxone®, 1 equiv.) in water, and mixture was stirred at r.t. for 2h (water/THF, 1:1, v/v). After completion of the reaction, it was partially concentrated *in vacuo* to 50% volume. The precipitate was filtered and washed with water and dried in oven. The crude product was crystallized from ethanol to afford light yellow crystals.

*3-(Methylsulfonyl)-6-phenyl-1,2,4-triazine (**7a**).* Yield: 66%. m.p. 180-183°C. IR (KBr, cm^{-1}): 3461, 3058 (aromatic C-H), 2916 (aliphatic C-H), 1598, 1537 (S=O). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.15 (s, 3H), 7.63-7.64 (m, 3H), 8.16-8.18 (m, 2H), 9.20 (s, 1H); MS: (m/z, %): 235.1 (M^+, 2), 219.1 (14), 128.1 (73), 102.1 (100), 77.1 (40).

*3-(Methylsulfonyl)-6-(p-tolyl)-1,2,4-triazine (**7b**).* Yield: 43%. m.p. 200-202°C. IR (KBr, cm^{-1}): 3399, 3065, 3002 (aromatic C-H), 2919 (aliphatic C-H), 1607, 1554 (S=O). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.48 (s, 3H), 3.13 (s, 3H), 7.42 (d, *J* = 8.0 Hz, 2H), 8.07 (d, *J* = 8.2 Hz, 2H), 9.16 (s, 1H); MS: (m/z, %): 249.1 (M^+, 2), 237.1 (10), 190.1 (11), 136.0 (100), 116.1 (100).

*6-(4-Methoxyphenyl)-3-(methylsulfonyl)-1,2,4-triazine (**7c**).* Yield: 83%. m.p. 144-145°C. IR (KBr, cm^{-1}): 3451, 3000 (aromatic C-H), 2967, 2931, 2838 (aliphatic C-H), 1605, 1578 (S=O). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.13 (s, 3H), 3.94 (s, 3H), 7.13 (d, *J* = 8.9 Hz, 2H), 8.16 (d, *J* = 8.9 Hz, 2H), 9.14 (s, 1H); MS: (m/z, %): 265.1 (M^+, 2), 249.1 (12), 174.1 (20), 158.1 (59), 132.1 (100).

*4-(3-(Methylsulfonyl)-1,2,4-triazin-6-yl)phenol (**7d**).* Yield: 60%. m.p. 195-197°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.01 (s, 3H), 7.00 (d, *J* = 8.7 Hz, 2H), 8.16 (d, *J* = 8.7 Hz, 2H), 9.52 (s, 1H), 10.30 (s, 1H); MS: (m/z, %): 251.9 (M^+, 2), 179.9 (26), 132.1 (33), 93.0 (49), 64.0 (100).

*6-(4-Bromophenyl)-3-(methylsulfonyl)-1,2,4-triazine (**7e**).* Yield: 85%. m.p. 189-191°C. IR (KBr, cm^{-1}): 3459, 3085, 3047 (aromatic C-H), 1586 (S=O). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.15 (s, 3H), 7.78 (d, *J* = 8.6 Hz, 2H), 8.06 (d, *J* = 8.6 Hz, 2H), 9.18 (s, 1H); MS: (m/z, %): 313.0 (M^+, 2), 315.0 (M+2, 2), 297.0 (9), 206.0 (41), 180.1 (100), 127.1 (98), 101.1 (57).

*6-(4-Chlorophenyl)-3-(methylsulfonyl)-1,2,4-triazine (**7f**).* Yield: 88%. m.p. 210-212°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.15 (s,3H), 7.61 (d, *J* =8.6 Hz, 2H), 8.14 (d, *J* =8.7 Hz, 2H), 9.18 (s, 1H); MS: (m/z, %): 269.1 (M^+, 0.5), 253.1 (8), 162.1 (46), 136.1 (100), 101.1 (19).

*General procedure for the preparation of 3-hydrazinyl-6-aryl-1,2,4-triazines **8a-f***

To a suspension of *3-(methylsulfonyl)-6-aryl-1,2,4-triazines **7a-f*** (1 mmol) in THF (11 ml) was added 0.53 ml of hydrazine monohydrate and the resulting mixture stirred at r.t. for 2h. The precipitated product was filtered and air dried.

*3-Hydrazinyl-6-phenyl-1,2,4-triazine (**8a**).* Yield: 86%. Yellow powder. m.p. 192-194°C. IR (KBr, cm^{-1}): 3275, 3218 (N-H), 3055 (aromatic C-H), 2965 (aliphatic C-H). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.46 (brs, 2H), 7.46 (t, *J* = 7.3 Hz, 1H), 7.52 (t, *J* = 7.5 Hz, 2H), 8.02 (d, *J* = 7.2 Hz, 2H), 8.87 (s, 1H); MS: (m/z, %): 187.2 (M^+, 80), 116 (13), 102.2 (100).

*3-Hydrazinyl-6-(p-tolyl)-1,2,4-triazine (**8b**).* Yield: 70%. Yellow powder. m.p. 173-174°C. . IR (KBr, cm^{-1}): 3432, 3233 (N-H), 3043 (aromatic C-H). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.36 (s, 3H), 4.47 (brs, 2H), 7.32 (d, *J* = 8.1 Hz, 2H), 7.91 (d, *J* = 8.2 Hz, 2H), 8.82 (brs, 1H), 8.85 (s, 1H); MS: (m/z, %): 201.2 (M^+, 84), 130.1 (8), 116.2 (100), 89.1 (12).

*3-Hydrazinyl-6-(4-methoxyphenyl)-1,2,4-triazine (**8c**).* Yield: 65%. Yellow powder. m.p. 213-215°C. IR (KBr, cm^{-1}): 3461, 3254 (N-H), 3039 (aromatic C-H), 2963, 2840 (aliphatic C-H). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.82 (s, 3H), 4.42 (s, 2H), 7.07 (d, *J* = 8.9 Hz, 2H), 7.97 (d, *J* = 8.8 Hz, 2H), 8.74 (brs, 1H), 8.83 (s, 1H); MS: (m/z, %): 217.2 (M^+, 72), 132.2 (100), 117.0 (38), 89.1 (32).

*4-(3-Hydrazinyl-1,2,4-triazin-6-yl)phenol (**8d**).* Yield: 64%. The yellow needle crystals were obtained by recrystallization from ethanol. m.p. 243-245°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.38 (brs, 2H), 6.88 (d, *J* = 8.6 Hz, 2H), 7.84 (d, *J* = 8.6 Hz, 2H), 8.67 (brs, 1H), 8.77 (s, 1H).

*6-(4-Bromophenyl)-3-hydrazinyl-1,2,4-triazine (**8e**).* Yield: 90%. Yellow powder. m.p. 228-230°C. IR (KBr, cm^{-1}): 3438, 3249 (N-H). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.48 (s, 2H), 7.71 (d, *J* = 8.5 Hz, 2H), 7.98 (d, *J* = 8.6 Hz, 2H), 8.89 (s, 1H), 8.96 (brs, 1H); MS: (m/z, %): 265.0 (M^+, 35), 267.0 (M+2, 35), 250.0 (5), 182.0 (100), 101.0 (52).

*6-(4-Chlorophenyl)-3-hydrazinyl-1,2,4-triazine (**8f**).* Yield: 80%. Yellow powder. m.p. 230-232°C. IR (KBr, cm^{-1}): 3301, 3224 (N-H), 3042 (aromatic C-H), 2969 (aliphatic C-H). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.48 (brs, 2H), 7.57 (d, *J* = 8.6 Hz, 2H), 8.05 (d, *J* = 8.6 Hz, 2H), 8.89 (s, 1H), 8.95 (brs, 1H).

*General procedure for the preparation of 6-aryl-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiols **9a-f***

To a suspension of *3-hydrazinyl-6-aryl-1,2,4-triazines **8a-f*** (1 mmol) in ethanol (15 ml), were added successively potassium hydroxide (2N, 1.3 ml) and carbon disulfide (1.3 ml) and the mixture was refluxed for 1 h. After completion, the reaction mixture was cooled to r.t. and concentrated *in vacuo*. 1 N aqueous potassium hydroxide was added to the residue, heated and sonicated. Then solution was filtered and the filtrate was acidified to pH 2-3 with 1 N aqueous HCl to precipitate the product. The resulting precipitate was filtered, washed with water and dried in oven. It was used in the next step without further purification.

*6-Phenyl-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiol (**9a**).* Yield: 88%. Light orange powder. m.p. 272-274°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.64-7.66 (m, 3H), 8.16-8.18 (m, 2H), 9.35 (s, 1H), 14.94 (s, 1H); MS: (m/z, %): 229.1 (M^+, 100), 197.1 (5), 116.1 (22), 104.1 (51), 89.1 (15).

*6-(p-Tolyl)-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiol (**9b**).* Yield: 60%. Light orange powder. m.p. 254-257°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.43 (s, 3H), 7.45 (d, *J* = 8 Hz, 2H), 8.09 (d, *J* = 8.2 Hz, 2H), 9.34 (s, 1H), 14.88 (s, 1H); MS: (m/z, %): 243.1 (M^+, 100), 229.1 (3), 155.1 (10), 130.1 (26), 118.1 (60), 91.1 (26).

*6-(4-Methoxyphenyl)-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiol (**9c**).* Yield: 82%. Light orange powder. m.p. 232-234°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.88 (s, 3H), 7.19 (d, *J* = 8.9 Hz, 2H), 8.16 (d, *J* = 8.9 Hz, 2H), 9.34 (s, 1H), 14.84 (s, 1H).

*4-(3-Mercapto-[1,2,4]triazolo[4,3-b][1,2,4]triazin-6-yl)phenol (**9d**).* Yield: 71%. Brick red powder. m.p. 260-262°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 6.99 (d, *J* = 8.7 Hz, 2H), 8.06 (d, *J* = 8.8 Hz, 2H), 9.30 (s, 1H), 14.81 (s, 1H).

*6-(4-Bromophenyl)-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiol (**9e**).* Yield: 73%. Light orange powder. m.p. 257-259°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.87 (d, *J* = 8.6 Hz, 2H), 8.12 (d, *J* = 8.6 Hz, 2H), 9.35 (s, 1H), 14.95 (s, 1H); MS: (m/z, %): 307.0 (M^+, 8), 309 (M+2, 8), 267.0 (100), 196 (69), 149.9 (36), 102.0 (31), 88.0 (24).

*6-(4-Chlorophenyl)-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiol (**9f**).* Yield: 90%. Light orange powder. m.p. 247-249°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.73 (d, *J* = 8.7 Hz, 2H), 8.20 (d, *J* = 8.7 Hz, 2H), 9.35 (s, 1H), 14.95 (s, 1H); MS: (m/z, %): 263.1 (M^+, 8), 219.1 (33), 168.0 (88), 149.0 (100), 136.0 (30), 89.1 (38).

General procedure for the preparation of final derivatives

To a suspension of 6-aryl-[1,2,4]triazolo[4,3-b][1,2,4]triazine-3-thiols ***9a-f*** (1 equiv.) and 1.1 equiv. of 3- or 4-(chloromethyl)pyridine hydrochloride in DMSO, was added 2.5 equiv. of potassium carbonate which was then stirred at r.t. for 3h. Chloroform was added to the resulting mixture and washed with water (×3), then dried over Na_2SO_4 and concentrated to give the crude product as a brown oil. Final products were purified by column chromatography (silica gel, 230–400 mesh) eluting with chloroform/methanol (1%).

*6-Phenyl-3-((pyridin-4-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**10a**)*

Yield: 30%; m.p. 130-132°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.59 (s, 2H), 7.38 (d, *J* = 5.9 Hz, 2H), 7-56-7.61 (m, 3H), 7.98 (dt, *J* = 1.5, 6.6 Hz, 2H), 8.50 (d, *J* = 5.8 Hz, 2H), 8.95 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 34.6, 124.1, 127.6, 129.8, 131.1, 132.3, 144.8, 145.7, 146.0, 148.2, 148.8, 150.2; MS: (m/z, %): 320.1 (M^+, 92), 287.1 (9), 229.1 (100), 190.1 (31), 104.1 (64), 77.1 (35). Anal. Calcd for $C_{16}H_{12}N_6S$: C, 59.98; H, 3.78; N, 26.23. Found: C, 59.89; H, 3.83; N, 26.29.

*3-((Pyridin-4-ylmethyl)thio)-6-(p-tolyl)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**10b**)*

Yield: 35%; m.p. 187-188°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.44 (s, 3H), 4.58 (s, 2H), 7.36-7.38 (m, 4H), 7.88 (d, *J* = 8.2 Hz, 2H), 8.50 (dd, *J* = 1.5, 6 Hz, 2H), 8.93 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.7, 34.6, 124.1, 127.4, 128.2, 130.5, 143.1, 144.7, 145.7, 146.1, 148.2, 148.8, 150.2; MS: (m/z, %): 334.1 (M^+, 100), 301.1 (10), 256.1 (8), 224.1 (8), 190.0 (47), 116.1 (47). Anal. Calcd for $C_{17}H_{14}N_6S$: C, 61.06; H, 4.22; N, 25.13. Found: C, 61.19; H, 4.24; N, 25.09.

*6-(4-Methoxyphenyl)-3-((pyridin-4-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**10c**)*

Yield: 37%; m.p. 148-150°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.88 (s, 3H), 4.56 (s, 2H), 7.05 (d, *J* = 8.9 Hz, 2H), 7.37 (d, *J* = 5.9 Hz, 2H), 7.94 (d, *J* = 8.8 Hz, 2H), 8.49 (d, *J* = 6 Hz, 2H), 8.91 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 34.6, 55.79, 115.3, 123.3, 124.1, 129.2, 144.6, 145.8, 146.0, 147.7, 148.8, 150.2, 163.0; MS: (m/z, %): 350.1 (M^+, 100), 317.1 (8), 215.1 (23), 190.0 (46), 146.1 (46), 132.1 (56). Anal. Calcd for $C_{17}H_{14}N_6OS$: C, 58.27; H, 4.03; N, 23.98. Found: C, 58.35; H, 4.05; N, 24.05.

*4-(3-((Pyridin-4-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazin-6-yl)phenol (**10d**)*

Yield: 15%; m.p. 211-213 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.45 (s, 2H), 6.89 (d, *J* = 8.5 Hz, 2H), 7.29 (d, *J* = 4.7 Hz, 2H), 7.94 (d, *J* = 8.6 Hz, 2H), 8.37 (brs, 2H), 9.22 (s, 1H), 10.28 (s, 1H) ;^{13}C NMR (125 MHz, DMSO-d_6) δ: 34.5, 116.8, 122.5, 124.5, 130.1, 143.2, 147.1, 148.3, 148.6, 149.3, 150.2, 161.6; MS: (m/z, %): 336.1 (M^+, 15), 187.1 (31), 119.0 (42), 93.1 (100). Anal. Calcd for $C_{16}H_{12}N_6OS$: C, 57.13; H, 3.60; N, 24.98. Found: C, 57.02; H, 3.66; N, 24.90.

*6-(4-Bromophenyl)-3-((pyridin-4-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**10e**)*

Yield: 30%; m.p. 158-160°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.53 (s, 2H), 7.32 (d, *J* = 5.6 Hz, 2H), 7.66 (d, *J* = 8.3 Hz, 2H), 7.81 (d, *J* = 8.3 Hz, 2H), 8.44 (d, *J* = 5.4 Hz, 2H), 8.85 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 34.5, 124.1, 127.4, 128.9, 129.9, 133.1, 145.0, 145.4, 145.6, 147.3, 148.6, 150.2; MS: (m/z, %): 398 (M^+, 58), 400 (M+2, 60), 367.0 (9), 334.1 (22), 215.1 (18), 190.0 (100), 92.1 (73). Anal. Calcd for $C_{16}H_{11}BrN_6S$: C, 48.13; H, 2.78; N, 21.05. Found: C, 48.22; H, 2.71; N, 21.12.

*6-(4-Chlorophenyl)-3-((pyridin-4-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**10f**)*

Yield: 20%; m.p. 151-153 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.54 (s, 2H), 7.33 (d, *J* = 4.9 Hz, 2H), 7.51 (d, *J* = 8.4 Hz, 2H), 7.88 (d, *J* = 8.4 Hz, 2H), 8.45 (d, *J* = 4.9 Hz, 2H), 8.68 (s, 1H); MS: (m/z, %): 354.1 (M^+, 100), 356.1 (M+2, 39), 321.1 (22), 263.0 (58), 190.0 (85), 163.0 (27), 136.0 (85), 92.1 (77). Anal. Calcd for $C_{16}H_{11}ClN_6S$: C, 54.16; H, 3.13; N, 23.69. Found: C, 54.25; H, 3.18; N, 23.61.

*6-Phenyl-3-((pyridin-3-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**11a**)*

Yield: 38%; m.p. 145-147 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.62 (s, 2H), 7.20 (dd, *J* = 4.8, 7.8 Hz, 1H), 7.56-7.61 (m, 3H), 7.82 (dt, *J* = 1.9, 7.8 Hz, 1H), 7.98 (dt, *J* = 1.7, 6.5 Hz, 2H), 8.46 (dd, *J* = 1.5, 4.7 Hz, 1H), 8.66 (d, *J* = 2.0 Hz, 1H), 8.95 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 33.2, 123.6, 127.6, 129.8, 131.1, 132.3, 132.6, 136.9, 145.0, 146.0, 148.2, 148.7, 149.3,150.3; MS: (m/z, %): 320.2 (M^+, 18), 287.1 (20), 242.1 (8), 190.1 (56), 116.1 (29), 92.1 (100). Anal. Calcd for $C_{16}H_{12}N_6S$: C, 59.98; H, 3.78; N, 26.23. Found: C, 60.09; H, 3.84; N, 26.16.

*3-((Pyridin-3-ylmethyl)thio)-6-(p-tolyl)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**11b**)*

Yield: 30%; m.p. 173-175°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.43 (s, 3H), 4.60 (s, 2H), 7.18 (dd, *J* = 4.8, 7.8 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 2H), 7.80 (dt, *J* = 1.8, 7.8 Hz, 1H), 7.86 (d, *J* = 8.2 Hz, 2H), 8.44 (d, *J* = 4.5 Hz, 1H), 8.64 (s, 1H), 8.92 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.7, 33.2, 123.6, 127.4, 128.2, 130.5, 132.6, 136.9, 143.1, 144.9, 146.1, 148.1, 148.8, 149.2, 150.3; MS: (m/z, %): 334.1 (M^+, 36), 301.1 (29), 256.1 (12), 215.1 (15), 190.0 (73), 130.1 (35), 116.1 (62), 92.1 (100). Anal. Calcd for $C_{17}H_{14}N_6S$: C, 61.06; H, 4.22; N, 25.13. Found: C, 61.14; H, 4.16; N, 25.06.

*6-(4-Methoxyphenyl)-3-((pyridin-3-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**11c**)*

Yield: 34%; m.p. 146-148°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.87 (s, 3H), 4.59 (s, 2H), 7.03 (d, *J* = 8.8 Hz, 2H), 7.18 (dd, *J* = 4.8, 7.7 Hz, 1H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.93 (dd, *J* = 1.8, 8.8 Hz, 2H), 8.43 (d, *J* = 4.7 Hz, 1H), 8.63 (s, 1H), 8.91 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 33.2, 55.7, 115.2, 123.3, 123.6, 129.2, 132.6, 136.9, 144.7, 146.0, 147.7, 148.7, 149.2, 150.3, 163.0; MS: (m/z, %): 350.1 (M^+, 42), 317.1 (23), 272.1 (12), 190.0 (62), 132.1 (75), 92.1 (100). Anal. Calcd for $C_{17}H_{14}N_6OS$: C, 58.27; H, 4.03; N, 23.98. Found: C, 58.32; H, 4.08; N, 23.90.

*4-(3-((Pyridin-3-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazin-6-yl)phenol (**11d**)*

Yield: 25%; m.p. 268-270°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.61 (s, 2H), 7.02 (d, *J* = 8.7 Hz, 2H), 7.33 (dd, *J* = 4.7, 7.7 Hz, 1H), 7.83 (dd, *J* = 1.5, 7.8 Hz, 1H), 8.07 (d, *J* = 8.7 Hz, 2H), 8.44 (d, *J* = 4 Hz, 1H), 8.59 (s, 1H), 9.33 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 32.5, 116.2, 121.8, 123.3, 129.4, 133.3, 136.4, 142.8, 147.7, 147.8, 148.4, 148.6, 149.8, 161.0; MS: (m/z, %): 336.1 (M^+, 2), 292.1 (9),174.1 (27), 119.0 (88), 92.1 (100). Anal. Calcd for $C_{16}H_{12}N_6OS$: C, 57.13; H, 3.60; N, 24.98. Found: C, 57.18; H, 3.56; N, 24.92.

*6-(4-Bromophenyl)-3-((pyridin-3-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**11e**)*

Yield: 37%; m.p. 176-178°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.63 (s,2H), 7.21 (dd, *J* = 4.8, 7.8 Hz, 1H), 7.72 (d, *J* = 8.6 Hz, 2H), 7.82 (dt, *J* = 1.9, 7.8 Hz, 1H), 7.87 (d, *J* = 8.6 Hz, 2H), 8.46 (dd, *J* = 1.5, 4.8 Hz, 1H), 8.67 (d, *J* = 2.1 Hz, 1H), 8.91 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 33.2, 123.6, 127.4, 128.9, 130.0, 132.5, 133.1, 136.9, 145.2, 145.4, 147.3, 148.6, 149.3, 150.3; MS: (m/z, %): 398 (M^+, 8), 400 (M+2, 8), 365.1 (8), 281.0 (4), 215.1 (14), 190.0 (63), 92.1 (100). Anal. Calcd for $C_{16}H_{11}BrN_6S$: C, 48.13; H, 2.78; N, 21.05. Found: C, 48.10; H, 2.84; N, 21.10.

*6-(4-Chlorophenyl)-3-((pyridin-3-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine (**11f**)*

Yield: 40%; m.p. 158-160°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.62 (s, 2H), 7.20 (dd, *J* = 4.9, 7.7 Hz, 1H), 7.55 (d, *J* = 8.6 Hz, 2H), 7.81 (dt, *J* = 1.8, 7.9 Hz, 1H), 7.94 (d, *J* = 8.6 Hz, 2H), 8.46 (d, *J* = 4.6 Hz, 1H), 8.66 (s, 1H), 8.91 (s, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ: 33.2, 123.6, 128.8, 129.5, 130.1, 132.5, 136.9, 138.9, 145.2, 145.4, 147.2, 148.6, 149.3, 150.3; MS: (m/z, %): 354 (M^+, 12), 356 (M+2, 4), 321.1 (12), 240.0 (13), 215.1 (22), 190.1 (46), 92.1 (100). Anal. Calcd for $C_{16}H_{11}ClN_6S$: C, 54.16; H, 3.13; N, 23.69. Found: C, 54.19; H, 3.17; N, 23.77.

X-ray crystallography

A crystal of compound **11c** was prepared by slow evaporation in acetonitrile and mounted under Paratone-N oil and flash cooled to 150 K in a stream of nitrogen in an Oxford Cryostream cooler. Single-crystal X-ray intensity data (Table 1) were collected using an Agilent Gemini S Ultra diffractometer (Cu Kα radiation (λ = 1.54180 Å)). The data were reduced within the CrysAlisPro software. [9] The structure was solved using the program Superflip [10] and all non-hydrogen atoms located. Least-squares refinements on *F* were carried out using the CRYSTALS suite of programs. [11] The non-hydrogen atoms were refined anisotropically. All hydrogen atoms were

located using difference Fourier maps. Each hydrogen atom connected to carbon was placed geometrically with a C-H distance of 0.95 Å and a *U*iso of 1.2 times the value of *U*equiv of the parent C atom. The positions of these hydrogen atoms were then refined with riding constraints. [12]

HGF-induced proliferation assay

The anti-proliferative activities of compounds and positive control crizotinib (Sigma, PZ0191) were evaluated against MCF-7, MDA-MB-231, HepG2 and A549 cell lines using MTT assay. The cancer cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, %1 L-glutamine and % 0.5 antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2. Cancer cells were seeded in 96-well plates at 5000 cells/well. After 24 hours' incubation, cells were starved in FBS-free medium containing 0.1% BSA and recombinant human HGF (40 ng/ml) (Sigma, H5791) for 24 h, then treated with test compounds at different concentrations for 72 hours at 37 °C. After incubation, 10 µL MTT reagent (5 mg/ml) was added to each well. MTT formazan crystals were dissolved by the addition of 100 µL DMSO. Relative cell viability was estimated by comparing the absorbance values at 690 nm (for absorbance of MTT formazan) and 570nm (for the reference wavelength) of the treated group and untreated control group using a Biotek Power Wave XS microplate reader. IC_{50} values were calculated using GraphPad Prism software. [13, 14]

Caspase 3/7 Activity Assay

MCF-7, MDA-MB-231, HepG2 and A549 cells were seeded at a density of 1 x 10^3 cells per well in Lumox (Sarstedt) 384-well plates. After cells were challenged with recombinant human HGF (40 ng/ml) (Sigma, H5791) for 24 h, the test compounds were added at 20 µM concentration and the cells were incubated for an additional 24 hours at 37°C. The ApoTox-Glo Triplex Assay (Promega, USA) was performed to estimate viability, cytotoxicity and caspase-3/7 activity following the manufacturer's instructions. Briefly 5 µL viability/cytotoxicity reagent were added to the wells and after 1 hour of incubation at 37°C, viability ($400_{ex}/505_{em}$) and cytotoxicity ($485_{ex}/520_{em}$) were measured. Later 25 µL Caspase-Glo reagent was added to the wells and the plate is incubated for an hour at room temperature. Luminescence was measured using a multimode plate reader (BMG Labtech Omega FLUOstar). Caspase 3/7 activity results were normalized well-to-well by the ratio of the number of live cells to the number of dead cells. [15]

c-Met inhibition assay

Met Kinase Enzyme System (V3361, Promega, USA) with ADP-Glo assay was used for the c-Met inhibition assay. It was performed in duplicate to screen kinase inhibitory activities of compounds at 20 µM, 5 µM, 0,5 µM, 0.05 µM and 0.5 nM concentrations according to the manufacturer's instructions. Briefly reactions were set up in 384-well Lumox (Sarstedt) plates containing kinase buffer, 10 µM ATP, 0.2 µg/µl poly (Glu, Tyr) 4:1 peptide substrate and 8 ng c-Met kinase for 1 hour at room tempature. ADP formed from kinase reactions were measured by ADP-Glo kinase assay. 5 µL of ADP-Glo™ reagent was added to the wells to stop the kinase reaction and plate is incubated for 40 min at room temperature. After adding 10 µL of kinase

detection reagent and 30 min of additional incubation; luminescence was measured using a multimode plate reader (BMG Labtech Omega FLUOstar). The percentage of inhibition (I%) was estimated by comparing the kinase activities of samples to vehicle. IC_{50} values of the compounds were determined from percent kinase inhibition values between 20 µM-0.5 nM compound concentrations using Graphpad Prism 5 software.

Molecular docking study

A docking study was performed employing the AutoDock 4.2 software with the AutoDock Tools (ADT) interface. Compounds ***10c*** and ***11c*** were docked into the binding site of c-met tyrosine kinase (PDB code: 3ZXZ). At first, for the preparation of protein, all the water and ligands were removed and the protein structure was optimized and the Kollman charge was computed. The compound's structure was then optimized and Gasteiger–Huckel charges were added. The Lamarckian genetic algorithm was utilized for the docking search. A grid box was generated by Autogrid 4.2. Autogrid and autodock files were created by the following parameters: Grid center: X = 19.775, Y = 83.506, Z = 4.432, Grid spacing = 0.375, number of points: 60 × 60 × 60, number of evaluations: 2,500,000, population size: 150, GA run: 30.

Results & Discussion

Chemistry

The synthetic procedure for the title compounds is depicted in Figure 3. Oxidation of acetophenones (**3a-f**) by using selenium dioxide in a mixture of 1,4-dioxane and water in refluxing mode afforded corresponding 2,2-dihydroxy-1-arylethanones (**4a-f**) [16]. Subsequently compounds (**4a-f**) were reacted with triethyl orthoformate in the presence of *p*-toluenesulfonic acid (PTSA) in dichloromethane under reflux to give corresponding acetal intermediates (**5a-f**) [7]. The triazine ring closure was carried out in three successive steps for compounds ***6a-c*** and ***6e-f***. At the first step, reaction of acetal intermediates (**5a-f**) with thiosemicarbazide in the presence of PTSA in ethanol at room temperature led to the imine formation, then, methylation of the thiosemicarbazide thiol group was performed using iodomethane, and finally 1,2,4-triazine ring closure was done in acetic acid [17]. In order to prepare compound **6d**, the acetal intermediate **5d** was reacted with methyl hydrazinecarbimidothioate hydroiodide in the presence of PTSA in ethanol and then stirred in acetic acid at 60°C [18]. Next, the 3-methylthio group was oxidized by oxone in tetrahydrofuran and water at room temperature to afford **7a-f** [19]. The 3-hydrazinyl intermediates (**8a-f**) were prepared by replacement of the 3-methylsulfonyl group with a hydrazine group [17] and eventually it was cyclized in the presence of carbon disulfide in

ethanol to give 3-mercapto-[1,2,4]triazolo[4,3-b][1,2,4]triazines **9a-f** [20]. Final products, **10a-f** and **11a-f**, were prepared by adding commercially available 3- or 4-(chloromethyl)pyridine hydrochloride to 3-mercapto-1,2,4-triazolo-1,2,4-triazines **9a-f** in dry DMSO and potassium carbonate [21]. Exploring ^{1}H NMR spectra of compounds **10a-f** and **11a-f** (NMR spectra in Supplementary material) shows that aliphatic SCH_2 protons appeared as singlet and are located at 4.45-4.59 and 4.59-4.63 ppm respectively. Notably, a singlet appeared at 8.85-8.95 ppm for compounds **10a-f** and **11a-f** is attributed to the C7-H proton of the triazine ring (Figure 2), except in **10d** and **11d,** where the same proton has been deshielded to 9.22 and 9.33 ppm, respectively. The ^{1}H-NMR spectra of compounds **10a-f** show 4 doublets in the aromatic part of each spectrum, of which two of them belong to the *para*-substituted aryl ring located at position 6 of triazolotriazine nucleus. Basically, the other two doublets in the form of an AB quartet at 7.3 and 8.4-8.5 ppm correspond to the pyridine ring for compounds **10a-f**. For example, the spectrum of *6-(4-bromophenyl)-3-((pyridin-4-ylmethyl)thio)-[1,2,4]triazolo[4,3-b][1,2,4]triazine* (**10e**) revealed two singlet signals in 8.85 and 4.53 ppm attributed to C-H in the triazolotriazine ring and S-CH_2, respectively. The AB quartet signals at 7.32 and 8.44 ppm correspond to pyridine ring protons and the AB quartet signals at 7.66 and 7.81 ppm are related to 4-bromophenyl ring protons. ^{13}C-NMR spectrum of **10e** displayed one signal at 34.5 ppm referring to S-CH_2, and 11 signals ranging from 124.1-150.2 ppm attributed to carbons in aromatic rings.

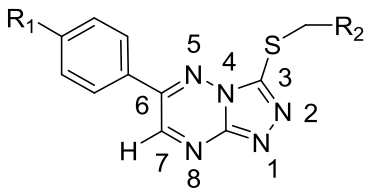


Figure 2. Atom numbering of [1,2,4]triazolo[4,3-b][1,2,4]triazine nucleus in IUPAC nomenclature.

In the ^{1}H-NMR spectra of compounds **11a-f**, the aryl ring substituted at position 6 of the triazolotriazine nucleus revealed two doublets in the form of an AB quartet, as observed for compounds **10a-f**. Generally, the pyridine ring in compounds **11a-f** shows a unique pattern as a doublet of doublets exists at 7.2 ppm, a multiplet (doublet of doublets or doublet of triplets) at 7.8, a doublet (or a doublet of doublets) at 8.4 and a singlet (or a doublet) at 8.6 ppm, and correctly confirm the presence of a pyridine-3-yl substructure. Specifically, in the ^{1}H-NMR

spectrum of compound **11e**, signals appeared at 7.73 and 7.87 ppm as a doublet corresponding to 4-bromophenyl ring protons. Signals at 7.20 (dd), 7.81 (dt), 8.46 (dd) and 8.66 (d) are assigned as pyridine-3-yl protons. Thus, signals at 4.63 and 8.91 ppm are related to aliphatic SCH_2 and triazine C7-H protons respectively. In the ^{13}C-NMR spectrum of **11e**, the aliphatic SCH_2 carbon atom appeared at 33.23 ppm and the remaining 13 aromatic carbon atoms are located in the range of 123.64-150.37 ppm and certainly confirms the correct structure of this compound.

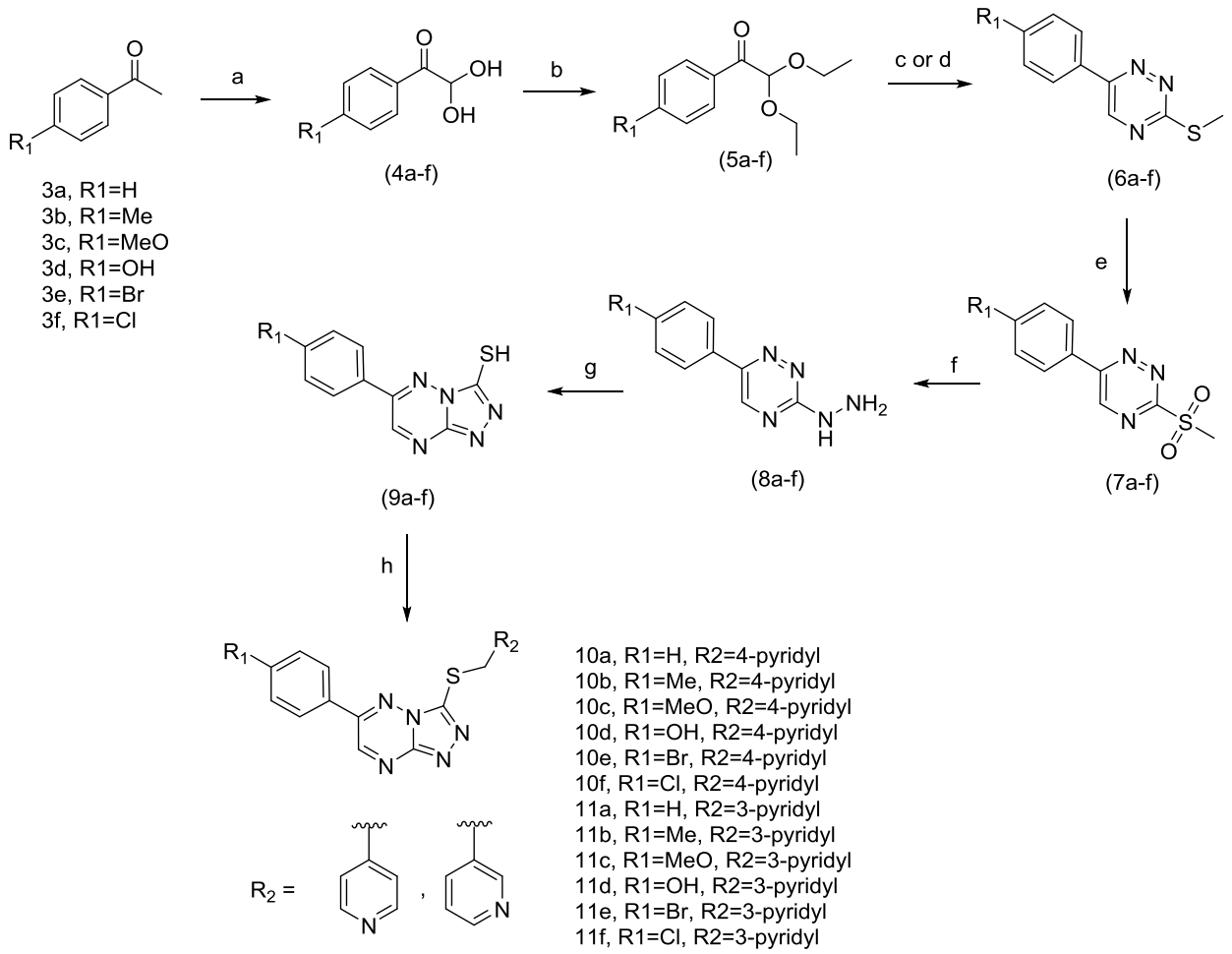


Figure 3. Synthetic scheme of triazolotriazines **10a-f** and **11a-f**. a) 1. SeO_2, 1,4-dioxane, reflux 2. H_2O, reflux b) Triethyl orthoformate, PTSA, CH_2Cl_2, reflux c) compounds ***6a-c*** and ***6e-f***: 1. Thiosemicarbazide, EtOH, PTSA, rt 2. CH_3I, stirr, rt 3. AcOH, 60°C d) compound ***6d***: methyl hydrazinecarbimidothioate hydroiodide, PTSA, EtOH, rt 2. AcOH,60°C, stirr e) Oxone, THF, H_2O, rt f) $NH_2NH_2.H_2O$, THF, rt g) CS_2, KOH, EtOH, reflux h) R_2CH_2Cl, K_2CO_3, DMSO, rt.

In vitro stability study

^{13}C-NMR spectroscopy was used to evaluate the stability of compound **11d** in a mixture of deuterated DMSO and chloroform. The solution of compound **11d** was stable at room temperature after 4 weeks and any signs of degradation or variations in chemical shifts were not observed (data in Supplementary material).

X-ray crystallography

X-ray crystallography was performed for a crystal of compound **11c** and the results are shown in Figure 4 and presented in Table 1. There is one molecule of **11c** in the asymmetric unit, giving rise to four molecules in the unit cell (Figure S1 in supplementary material). The molecules pack together in a zig zag fashion along the c axis (Figure S2 in supplementary material). A series of π-π interactions between the aromatic rings in adjacent molecules serve to hold the packed structure together. For example, the distance of the centroid of ring C(10), C(11), C(12), C(13), C(14), C(15) to the plane in the adjacent molecule containing the ring N(2), N(3), N(6), C(4), C(5), C(7) is 3.426(2) Å, and the distance of the centroid of the ring N(2), N(3), N(6), C(4), C(5), C(7) to this plane is 3.276(2) Å. Also the distance of the centroid of the ring N(20), C(18), C(19), C(21), C(22), C(23) to the plane in the adjacent molecule containing the ring N(20), C(18), C(19), C(21), C(22), C(23) is 3.432(2) Å. Notably, the distance of the centroid of the ring N(2), N(3), N(6), C(4), C(5), C(7) to the plane in the adjacent molecule containing the ring N(2), N(8), N(9), C(1), C(7) is 3.245(2) Å. (Figure S3 in supplementary material) Data have been deposited at the Cambridge Crystallographic Data Centre, deposit code: CCDC 1853261**.**

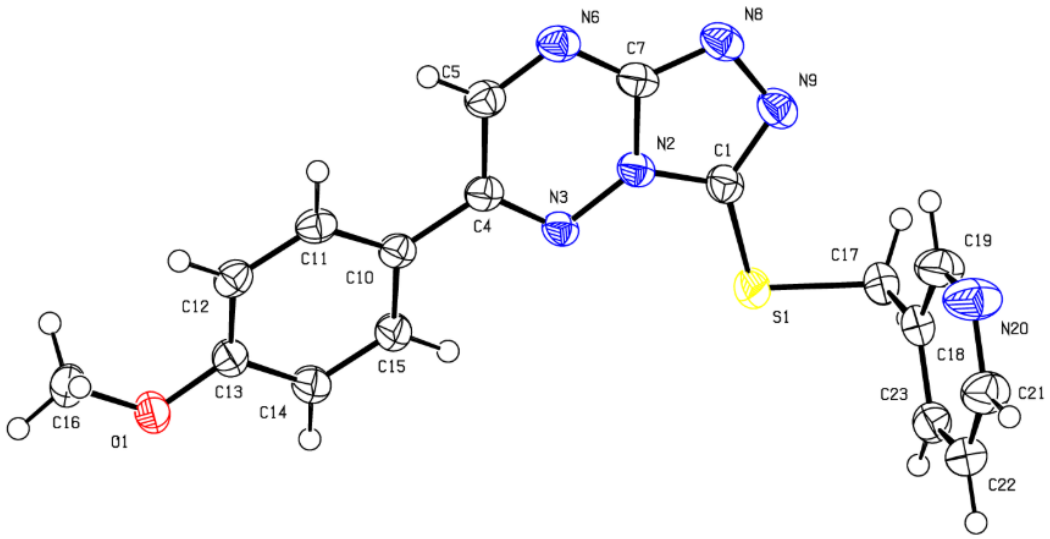


Figure 4. ORTEP structure of compound **11c**; Asymmetric unit with labelling scheme.

Table 1. Crystallographic data for **11c** at 150 K.

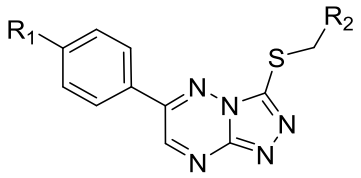
Formula	$C_{17}H_{14}N_6O_1S_1$
M_r	350.40
Crystal system	monoclinic
Space group	$P\,2_1/n$
Z	4
a/Å	4.77748(8)
b/Å	18.6830(5)
c/Å	17.9296(5)
β/°	94.1914(18)
V/Å^3	1596.07(7)
ρcalc/g cm^{-3}	1.458
Crystal habit	yellow plate
Crystal dimensions /mm	0.03 x 0.06 x 0.21
Radiation	Cu Kα
T/K	150
μ/mm^{-1}	1.967
No. Reflections ($I \leq 3\sigma(I)$), no. parameters	2684, 226
R(F), Rw(F)	0.0300, 0.0399

HGF-induced proliferation assay

The inhibitory effect of compounds on HGF-induced cell proliferation was evaluated against four cancer cell lines, including A549, HepG2 and MCF7 as high c-Met expressing cell lines, and against MDA-MB-231 as a low c-Met expressing cell line and the results are summarized in Table 2. Crizotinib, as a selective c-Met kinase inhibitor, was used as a reference drug in this study and the cancer cells were treated with recombinant human Hepatocyte Growth Factor (HGF) to induce c-Met kinase expression and activation. As the data in Table 2 indicate, most compounds exhibited good to excellent cytotoxicity with IC_{50} values ranging from 0.71 to 24.29 µM against four cancer cells. In general, all target compounds were more potent against A549 and HepG2 compared to the other two cell lines, MCF7 and MDA-MB-231. IC_{50} values of the target compounds on A549 range from 0.74 to 5.25 µM, but none of them showed more potency than the reference drug crizotinib (0.32 µM). The most potent compound on A549 cell line was determined to be compound **11b,** with an IC_{50} value of 0.74 µM. Interestingly, the data on HepG2 are the most promising since IC_{50} values range from 0.71 to 3.25 µM and two of the compounds displayed more cytotoxicity than crizotinib. Notably, compounds **10b** and **11e** with IC_{50} values of 0.74 and 0.71 µM respectively on HepG2 cells, showed 2.1 and 2.2-fold more potency than crizotinib (IC_{50} = 1.57 µM). Moreover, the target compounds showed acceptable cytotoxicity on MCF7 cancer cell lines and their IC_{50} values were in the range 9.46-21.17 µM. None of the target molecules were more cytotoxic than crizotinib (IC_{50} = 3.028 µM) on MCF7 cells but again compound **10b** was shown to have more cytotoxicity than the others with an IC_{50} value of 9.46 µM. The target compounds exhibited mild to moderate potency on MDA-MB-231 as a low expressing c-Met cell line. In all, HepG2 was the most sensitive cancer cell line against target molecules **10a-e** and **11a-e**. Substituent variations on the 6-aryl ring by methyl, methoxy, hydroxyl, bromine and chlorine groups displayed minimal impact on the cell proliferation results, so that no correlation could be found to relate structure and activity of these compounds. This finding can also be confirmed by docking calculations, in which the 6-aryl ring is oriented towards the solvent accessible region and there is not any contact between groups attached to the

6-aryl ring and the c-Met active site. There is no improvement or significant difference in the biological effects of the 4-pyridyl- and 3-pyridyl linked triazolotriazine compounds. On the other hand, the type of substitution of the pyridine ring has not changed the IC_{50} values on all cell lines significantly. This fact is clarified and discussed in the docking part of the discussion.

Table 2. Structure and IC_{50} values of compounds **10a-f** and **11a-f** in HGF-induced cell proliferation assay.



Compd.	R_1	R_2	IC_{50} (μM)			
			A549	HepG2	MCF7	MDA-MB-231
10a	H	4-pyridyl	2.02±0.03	1.27±0.02	16.43±1.09	9.65±1.15
10b	Me	4-pyridyl	1.14±0.09	**0.74±0.07***	9.46±1.10	24.29±1.20
10c	MeO	4-pyridyl	5.20±0.05	1.10±0.03	9.64±1.06	6.09±1.05
10d	OH	4-pyridyl	4.68±0.04	1.27±0.02	10.07±1.06	11.15±1.06
10e	Br	4-pyridyl	5.05±0.04	1.27±0.03	10.75±1.07	6.36±1.13
10f	Cl	4-pyridyl	5.25±0.03	1.47±0.02	21.17±1.21	16.18±1.15
11a	H	3-pyridyl	3.11±0.04	1.11±0.02	19.47±1.10	11.16±1.12
11b	Me	3-pyridyl	**0.74±0.05**	1.02±0.03	9.76±1.06	11.55±1.06
11c	MeO	3-pyridyl	1.76±0.04	3.25±0.04	10.19±1.03	10.07±1.07
11d	OH	3-pyridyl	1.43±0.04	2.11±0.07	10.19±1.05	11.91±1.06
11e	Br	3-pyridyl	2.33±0.06	**0.71±0.04***	12.55±1.09	11.59±1.07
11f	Cl	3-pyridyl	2.78±0.06	1.01±0.02	17.89±1.08	9.48±1.09
Crizotinib			0.32±0.06	1.57±0.03	3.028±1.06	1.99±1.08

The data are presented as mean±SEM and each experiment was performed in triplicate.
Bold values show the IC_{50} values of the target compounds lower than the values of crizotinib (also marked by an asterisk) or the most promising compounds in each column.

Caspase 3/7 Activity Assay

Caspases pertain to cysteine-aspartic proteases, promote controlled cell death or apoptosis. They modulate both intrinsic and extrinsic apoptotic pathways. Activation of caspases are mediated by proteolytic cleavage and finally leading to cell death during the apoptotic process. Impairment of apoptosis contribute in cancer development and progression. According to studies, the amount of caspases in cancer cells is reduced compared to normal cells.

The compounds were tested at 20 µM for 24 hours for viability, cytotoxicity and caspase 3/7 activity which is a marker of apoptosis in MCF7, MDA-MB-231, HepG2 and A549 cells and the data are illustrated in Figure 5. Accordingly, the compounds showed high cytotoxicity while caspase 3/7 activity was decreased. These results suggest that compounds are fast acting necrotic agents within 24 h and do not induce apoptosis through caspase 3/7 activity. These data also confirmed the results obtained by MTT assay (Table 2) in which target compounds had the most cytotoxic effect on HepG2 cells.

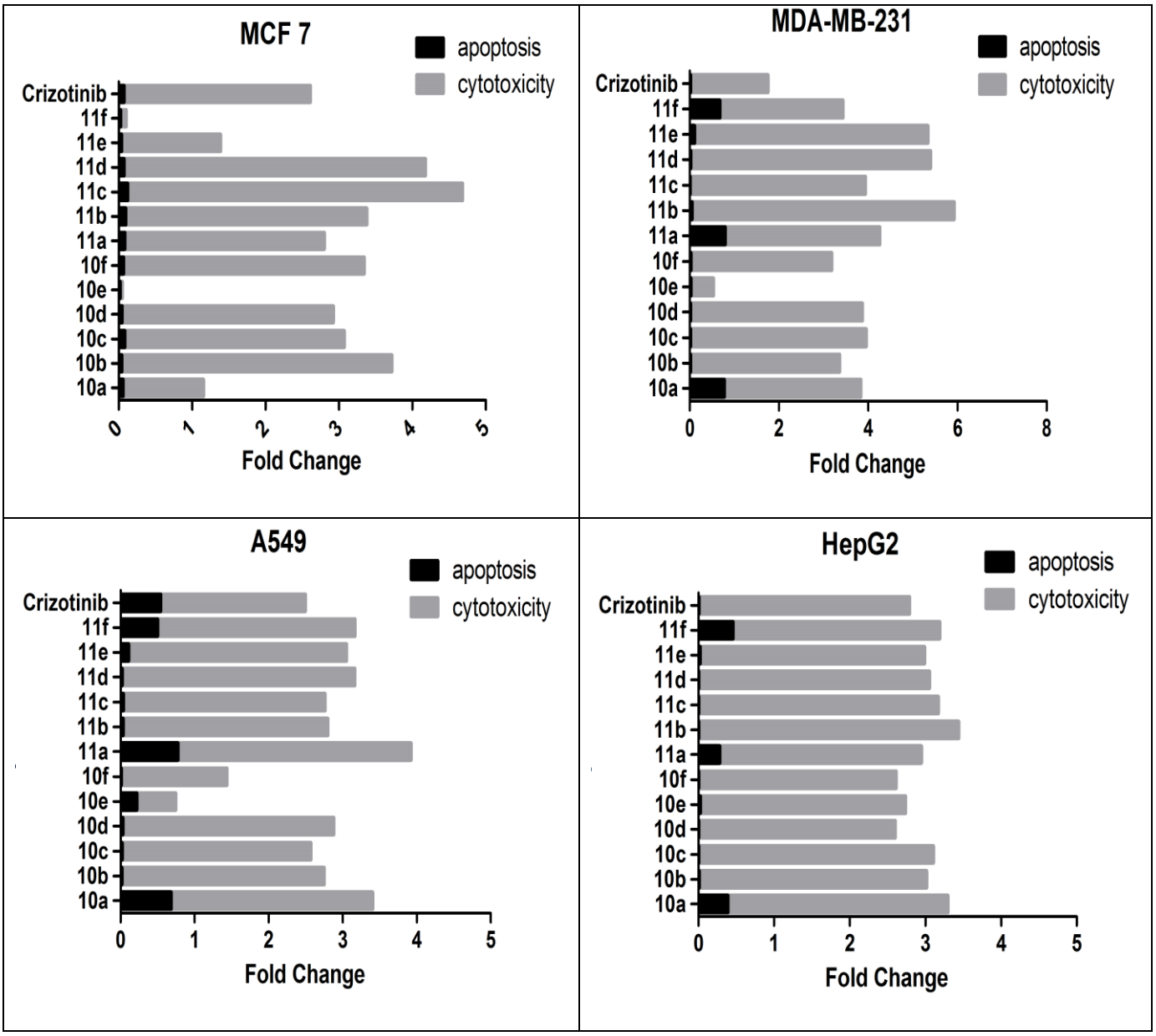


Figure 5. Extent of cytotoxicity and apoptosis mediated by Caspase 3/7 activity on MCF7, A549, HepG2 and MDA-MB-231 cell lines. Target compounds were tested at 20 µM concentration.

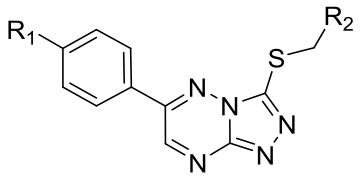
c-Met kinase inhibition assay

All compounds displayed significant c-Met kinase inhibition potential which were more than the values of crizotinib at 5, 0.5, 0.05 µM and 0.5 nM concentrations and the data are summarized in Table 3, as percent inhibition of c-Met kinase activity. All compounds showed complete inhibition of c-Met kinase activity at 20 µM, while crizotinib has 91% inhibition of c-Met kinase activity at this concentration (data not shown). At 5 µM concentration, all compounds had higher than 90% inhibition of c-Met, while compounds **10a** and **11d** showed the highest percentage inhibitions of 96.8% and 97.7%, respectively, which is higher than crizotinib (92.0%). At 0.5 µM concentration of target compounds, the percentage inhibition of c-Met was evaluated to be more than 80% for all compounds and interestingly, compounds **10a** (85.9%), **10b** (84.2%), **11b** (84.4%), **11e** (87.7%) and **11f** (86.1%) were shown to be more potent than crizotinib (83.0%) at this concentration. Surprisingly, the evaluation of c-Met kinase activity at lower concentration (0.05 µM) of target compounds, showed that compounds **10a** (79.3%), **10b** (80.8%), **10c** (77.6%), **10e** (77.9%), **11b** (80.9%), **11e** (79.1%) and **11f** (81.4%) have more inhibition potential of c-Met kinase activity than crizotinib (76.0%). Finally at 0.5 nM concentration, all the compounds exhibited a higher percent inhibition of c-Met kinase activity than crizotinib. Interestingly, all compounds displayed IC_{50} values in the range of 3.9-11.1 nM for c-Met kinase inhibition, clearly confirming that all compounds are more potent than crizotinib (IC_{50} = 11.1 nM).

As the data in Table 3 indicate, inclusion of the substituted pyridine ring (3- or 4-pyridyl) has not induced a significant or regular difference in potency between compounds **10a-f** and their equivalent derivatives **11a-f**. Correspondingly, the potency difference between the compounds is largely dependent on the type of substituted group at the para position of the phenyl ring. On the other hand, methyl- and bromine-substituted compounds **10b, 10e, 11b** and **11e** are the most potent ones in this series.

The obtained results confirmed our original hypothesis about high potency of thiomethylpyridine linked triazolotriazines to inhibit c-Met kinase activity and successfully suggest that all compounds and especially **10b** are recommended drug candidates for further in vivo and in vitro evaluations in the future.

Table 3. Percent inhibition of c-Met kinase activity by target compounds **10a-f** and **11a-f** at four different concentrations.



Compd.	R1	R2	% c-Met inhibition±SEM				IC_{50} (nM)
			5 µM	0.5 µM	0.05 µM	0.5 nM	
10a	H	4-pyridyl	96.8±0.9	**85.9±3.35**	**79.3±1.7**	**19.0±1.7**	6.2±0.07
10b	Me	4-pyridyl	93.9±1.3	**84.2±4.62**	**80.8±1.8**	**23.8±3.6**	4.6±0.10
10c	MeO	4-pyridyl	94.6±3.2	81.1±5.32	**77.6±1.9**	**17.1±3.1**	8.0±0.12
10d	OH	4-pyridyl	95.8±1.7	83.3±2.44	74.4±1.2	**23.0±2.5**	6.5±0.07
10e	Br	4-pyridyl	92.1±2.3	81.1±3.37	**77.9±3.0**	**29.6±1.7**	3.9±0.10
10f	Cl	4-pyridyl	93.1±1.9	83.5±1-17	74.8±3.4	**12.1±2.9**	11.1±0.08
11a	H	3-pyridyl	96.8±1.4	83.2±2.42	73.9±2.4	**16.6±2.3**	9.3±0.07
11b	Me	3-pyridyl	93.5±1.0	**84.4±4.58**	**80.9±1.6**	**20.3±3.5**	5.6±0.10
11c	MeO	3-pyridyl	94.4±1.0	80.0±2.84	71.7±3.5	**23.0±2.9**	7.8±0.09
11d	OH	3-pyridyl	97.7±0.8	79.5±2.32	75.4±2.8	**14.6±1.7**	10.1±0.09
11e	Br	3-pyridyl	90.2±1.3	**87.7±2.02**	**79.1±3.3**	**18.2±1.0**	6.4±0.08
11f	Cl	3-pyridyl	92.4±1.4	**86.1±2.54**	**81.4±2.9**	**15.4±1.0**	6.7±0.08
Crizotinib			92.0±2.2	83.0±2.61	76.0±1.7	11.4±1.4	11.1±0.09

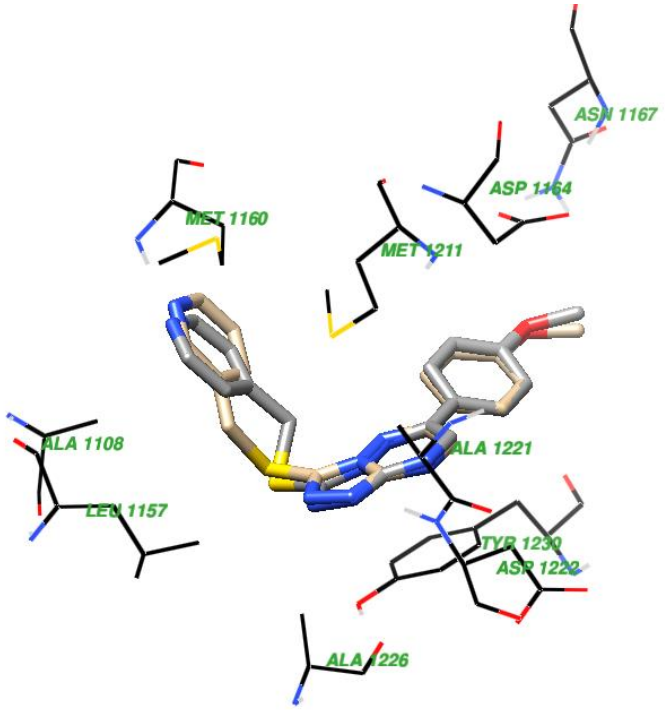
Bold values are %inhibition values higher than the value of crizotinib.

Molecular docking study

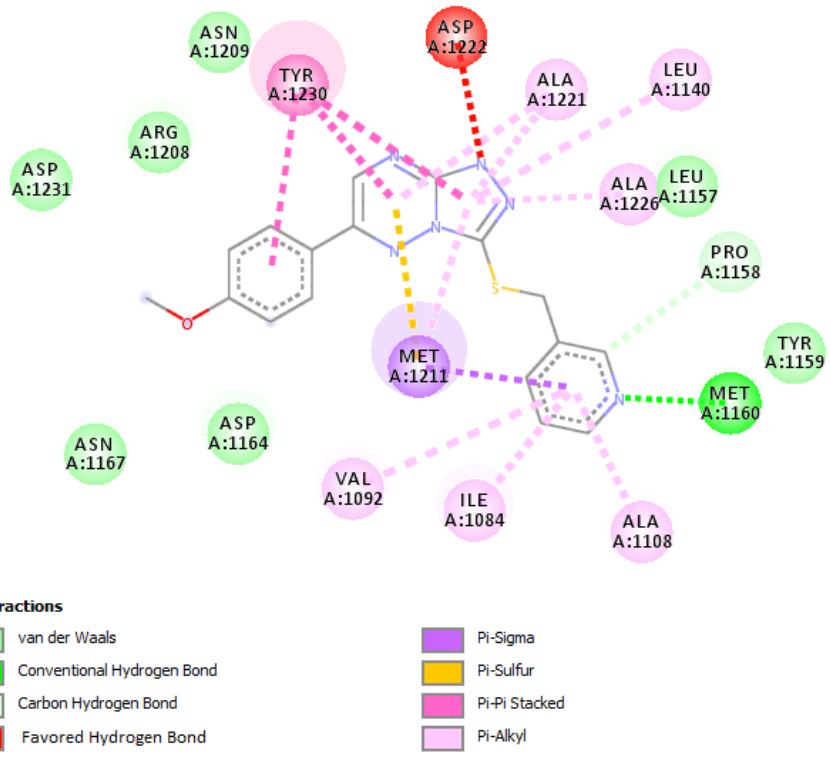
To explore the binding mode of the target compounds and to reach conclusions about the structure-activity relationship, molecular docking was performed for the two compounds, **10c** and **11c** as 4- and 3-pyridyl-linked triazolotriazines, respectively. Accordingly, PDB code 3ZXZ was used for an *in silico* study and our docking protocol was validated by the docking of co-crystal ligand PF-04217903 into the ATP-binding site of c-Met tyrosine kinase. The results of docking for the two compounds, **10c** and **11c,** are presented in Figure 6, and Figures S4 and S5

in the Supplementary material. As illustrated in Figure 6, both compounds have a very similar binding conformation so that the two compounds are almost superimposed on each other. Essentially, the triazolotriazine ring forms a hydrogen bond with Asp1222 and a π-π stacking interaction with Tyr1230, which are important in the exquisite c-Met kinase selectivity and potency. The pyridyl moiety occupies the hinge region and its nitrogen atom forms a crucial hydrogen bond with Met1160. The presence of a hydrogen-bond acceptor atom at this region of inhibitor has been implied in many SAR studies of c-Met kinase inhibitors [22]. As shown in Figures S4 and S5, this hydrogen bond has distances of 1.94 and 1.89 Å, respectively for compounds **10c** and **11c**. Furthermore, π-π stacking interaction occurs between Tyr1230 and *p*-methoxyphenyl ring. There are also several pi-alkyl interactions between bound inhibitors and aliphatic amino acids in the active site such as the alanine, valine and leucine residues.

a)


ASN 1167
ASP 1164
MET 1160
MET 1211
ALA 1108
ALA 1221
LEU 1157
TYR 1230
ASP 1222
ALA 1226

b)


ASN A:1209
ASP A:1222
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ALA A:1221
LEU A:1140
ARG A:1208
ASP A:1231
ALA A:1226
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PRO A:1158
TYR A:1159
MET A:1211
MET A:1160
ASP A:1164
ASN A:1167
VAL A:1092
ILE A:1084
ALA A:1108
Interactions
van der Waals
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Favored Hydrogen Bond
Pi-Sigma
Pi-Sulfur
Pi-Pi Stacked
Pi-Alkyl

Figure 6. a) Binding conformation of compounds **10c** (gray) and **11c** (brown) in the ATP-binding site of c-Met kinase. b) 2D map of bound conformation of **11c** in the ATP-binding site of c-Met kinase.

Conclusion

In summary, a series of compounds for use as c-Met kinase inhibitors were designed, synthesized and characterized by NMR, Mass spectrometry and X-ray crystallography. Their cytotoxicity was evaluated on A549, HepG2, MCF7 and MDA-MB-231 cancer cell lines. Most of the target compounds exhibited good to excellent cytotoxicity on A549 and HepG2 cell lines with IC_{50} values ranging from 0.71 to 5.25 µM. Interestingly, compounds **10b** and **11e** displayed more cytotoxicity than the reference drug crizotinib on HepG2 cells with IC50 of 0.74 and 0.71 µM respectively. Evaluation of c-Met kinase activity of target compounds showed that all compounds are more potent than crizotinib with IC50 values in the range of 3.9-11.1 nM. Finally, compound **10b** was introduced as a lead compound with IC_{50} value of 4.6 nM for c-Met kinase activity.

Future Perspective

Due to the emerge of new cancer types and resistance phenomenon to antitumor drugs, key biologic macromolecules involved in cancer progression have attracted much attention in drug discovery. c-Met tyrosine kinase receptor is one of the crucial targets responsible for tumor progression and migration and several studies have disclosed the triazolotriazines as a privileged scaffold for c-Met kinase inhibition. In the current study, novel triazolotriazine analogs were introduced as new leads for future drug development process as potent c-Met kinase inhibitors.

Summary Points

Design & synthesis of target compounds

- A series of compounds were designed, synthesized and biologically evaluated as c-Met tyrosine kinase inhibitors.
- Target compounds were characterized by ^{1}H and ^{13}C-NMR, Mass spectrometry and X-ray crystallography.

Biological studies

- Compounds **10b** and **11e** were more cytotoxic than crizotinib on HepG2 cells with IC_{50} values of 0.74 and 0.71 μM respectively.
- All of the compounds were more potent than crizotinib in c-Met kinase inhibition assay.
- Compound **10b** was introduced as a lead compound with IC_{50} of 4.6 nM for c-Met kinase.

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** this article implies the importance of hydrogen bondong of an h-bond acceptor with Met1160 in the active site.