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Original research paper

Design and development of novel 1,2,3-triazole chalcone derivatives as potential anti-osteosarcoma agents *via* inhibition of PI3K/Akt/mTOR signalling pathway

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ABSTRACT

Osteosarcoma (OS) is an uncommon tumour that mainly affects bone in children and adolescents. The current treatment options of OS are of limited significance due to their immense side effects. In the present manuscript, we have developed a novel series of 1,2,3-triazole chalcone derivatives as potential agents against OS. The compounds were synthesized and evaluated for their PI3K and mTOR inhibitory activity using luminescent kinase assay, and Lance ultra assay, resp. The entire set of compounds showed significant to moderate inhibition of both kinases in the nanomolar range. The three most active compounds: **4e** (*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)acryloyl)phenyl)-4-nitrobenzamide), **4f** (*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)acryloyl)phenyl)-4-chlorobenzamide) and **4g** (4-bromo-*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)acryloyl)phenyl)benzamide), were evaluated for anticancer activity against human OS cancer cell line (MG-63), liver cancer cell line (HepG2), lung cancer cell line (A549) and cervical cancer (HeLa), using MTT assay. Among the tested series, compound **4e** showed a better inhibitory profile than gedatolisib against PI3K and was approximately comparable to that of gedatolisib against mTOR. The most significant inhibitory activity was observed for compound **4e** against all cell lines (MG-63, HepG2, A549 and HeLa), still somewhat lower to comparable to that of gedatolisib, but with the highest potency against MG-63 cells. Compound **4e** was further tested for anticancer activity against other OS cells and showed to be equipotent to gedatolisib against U2OS and Saos-2 cells. Moreover, it was also found non-toxic to normal cells (BEAS-2B and MCF 10A). The effect of compound **4e** was further determined on apoptosis of Saos-2 cells by Annexin-PI assay, where it significantly amplified the percentage of apoptotic cells. Novel 1,2,3-triazole chalcone derivatives are potential agents against OS.

Keywords: 1,2,3-triazole-chalcone hybrids, osteosarcoma, kinase inhibition

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Production of osteoid or immature bone by the malignant cells causes the development of cancer of bone which is known as osteosarcoma (OS). It is a very uncommon tumour and majorly affects bone in children and adolescents. Each year nearly 750 to 900 new cases of OS are reported alone in the United States (1). In earlier days, surgical resection was the only option to treat OS which showed a low prognosis. However, the involvement of postoperative adjuvant chemotherapy along with surgical procedures has shown remarkable progress in the overall survival of the patients. The current therapeutic regime to treat OS relies on multi-drug chemotherapy consisting mainly of methotrexate (MTX), adriamycin (ADM), and cisplatin (DDP), with or without ifosfamide (2). Studies have shown that these drugs are coupled with serious adverse effects which limit their clinical use, such as myelosuppression, cardio-toxicity (anthracycline, antibiotics), bladder toxicity (ifosfamide), and gastrointestinal reactions, which typically manifest as nausea and vomiting (3). Therefore, the search for new compounds to treat OS with higher potency is urgently needed.

The aberrant activation of kinases has been considered as a major underlying cause for various cancers (4). Many compounds have been pursued to target a variety of kinases, such as CDK (5), EGFR (6), VEGFR (7), Hsp90 (8), *etc*. PI3K and mTOR inhibitors have found a significant place in anticancer therapeutics and many of them are now under various stages of drug development (9, 10).

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signalling pathways regulate cell proliferation, growth, cell size, metabolism, and motility of cells (9). Upon activation by various growth factor/ligands specific to receptor tyrosine kinase, PI3K phosphorylates PIP2 (phosphatidylinositol 4,5-bisphosphate) and converts it to PIP3 (phosphatidylinositol 3,4,5-triphosphate). This second messenger, PIP3, acts as a docking site for Akt and results in the phosphorylation of Akt (pAKt) at thr308 which encourages protein synthesis, cell growth, cell survival and cellular motility, by stimulating the downstream effector mTOR *via* TSC1/2 (tuberous sclerosis 1/2 complex) and upregulating various transcription factors (11). On the other hand, activated mTORC2 (mTOR complex 2) phosphorylates Akt at ser473 also resulting in hyperactivation of Akt (12, 13). Inhibition of this pathway has been shown to lead to regression of human tumours and some inhibitors of this pathway are approved by the FDA for clinical use, such as duvelisib, copanlisib and idelalisib (13–15).

A recent complementary genomic and pathway analysis identified PI3K/mTOR pathway aberrations in a subset of osteosarcoma samples (16). Based on this information, two dual PI3K/mTOR inhibitors (GSK2126458, BEZ-235) and a PIK3CA-specific inhibitor (PIK75) were tested against human- and murine-derived cell lines. All three drugs inhibited cell proliferation in all cell lines; PIK75 and GSK2126458 induced apoptosis as demonstrated by caspase 3/7 activation and poly(ADP-ribose) polymerase (PARP) cleavage (17). A recent report from the French Sarcoma Group of off-label use of targeted therapies for osteosarcoma found that those who received rapamycin (with or without cyclophosphamide) compared to a group of tyrosine kinase inhibitors (sunitinib, sorafenib, and pazopanib) had a superior progression-free survival (PFS) (18). Therefore, in view of the scarcity of active agents available for the treatment of recurrent and refractory osteosarcoma, inhibition of PI3K and mTOR may present a viable treatment strategy deserving the clinical investigation.

1,2,3-Triazole containing organoplatinum compounds showed significant activity against OS cells (MG-63) (19). The study also showed that 1,2,3-triazole-coumarin hybrids inhibit proliferation and promote apoptosis by sub-G1 phase arrest of the cell cycle in MG-63 cells (20). On the other hand, *trans*-chalcone, a chalcone derivative, inhibits the growth of human osteosarcoma cell lines by modulating Sp1 down-regulation at the transcriptional level and up-regulating p53 expression at the post-translational level (21). In another study, flavokawain B, a kava chalcone, inhibits the growth of human osteosarcoma cells through G2/M cell cycle arrest and apoptosis (22). Two novel chalcone derivatives, 4′-amino--1-naphthyl-chalcone (d14) and 4′-amino-4-methyl-1-naphthyl-chalcone (d15) suppress migration and invasion of OS cells mediated by p53 regulating EMT-related genes (21). 1,2,3-Triazole-chalcone hybrids are well known for their diverse array of pharmacological properties, for instance, antibacterial, anticancer, anti-HIV, anti-inflammatory and anti-tubercular (23, 24). In particular, they have shown significant inhibitory activity against various cancers, such as lung, breast, leukaemia, melanoma (25). Despite the immense significance of PI3K/mTOR in OS, till now, no single study has developed 1,2,3-triazole--chalcone as PI3K/mTOR inhibitor, to act as an anticancer agent against OS. Prompted by the above, in the present study, we intend to devise and develop novel 1,2,3-triazole--chalcone hybrids as putative inhibitors against OS and to study their mechanism of action.

EXPERIMENTAL

Chemistry

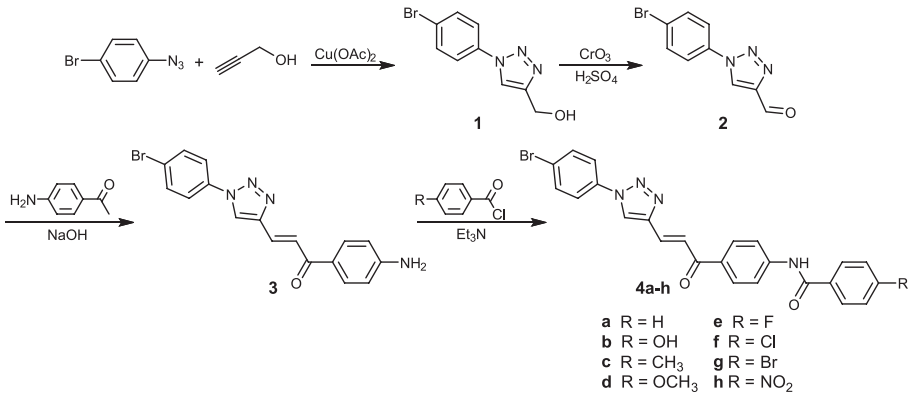
The chemicals and solvents were procured from Sigma-Aldrich (USA) and used without further purification. Mettler Toledo (USA) melting point apparatus (MP70) was used to record the melting points of compounds in sealed tubes. Thin-layer chromatography was conducted on aluminium TLC plates; silica gel was pre-coated with fluorescent indicator F254 (Sigma-Aldrich, USA). RX-I spectrophotometer (PerkinElmer, USA) was used to record IR spectra (in KBr, 2.0 cm^{-1}, flat, smooth) in the range of 4000–400 cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 (USA) at 400 MHz and 100 MHz, resp., using dimethyl sulfoxide (DMSO-d_6) as solvent and TMS as internal standard. Chemical shifts are shown in δ ppm. VG Auto Spec 3000 spectrometer (Waters, USA) with electrospray ionization (ESI) was used for recording mass spectra. The source conditions for EI were: accelerating voltage 7 kV and temperature 250 °C. Elemental analysis was performed on a 2400 CHNSO PerkinElmer analyzer.

Synthesis of compounds **1**, **2** and **3** was performed as previously described (26). Initially, the synthesis has been started by stirring 1-azido 4-bromobenzene (0.5 mmol) and prop-2-yn-1-ol in *tert*-butyl alcohol, in the presence of $Cu(OAc)_2$ which acts as a catalyst, under click reaction conditions, to afford compound **1** (1-(4-bromophenyl)-1*H*-1,2,3-triazol--4-yl)methanol)]. Furthermore, compound **1** was oxidised to the reaction intermediate, aldehyde, 1-(4-bromophenyl)-1*H*-1,2,3-triazole-4-carbaldehyde (**2**). Namely, compound **1** (0.5 mmol) was reacted with Jones reagent (CrO_3, aq. H_2SO_4, 3 mL) to afford compound **2**. In the second step, compound **2** (0.5 mmol) reacted with 1-(4-aminophenyl)ethanone (0.5 mmol) in ethyl alcohol, using aq. NaOH (10 %), to furnish (*E*)-1-(4-aminophenyl)-3-(1--(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)prop-2-en-1-one (**3**) in good yield (Tables SI and SII).

General procedure for the synthesis of target compounds **4a-h** *(26, 27)*

To a solution of compound **3** (1-(4-aminophenyl)-3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)prop-2-en-1-one) (1.0 mmol in 10 mL of anh. DCM), triethylamine (2.5 mmol) and the corresponding acyl chloride (2.5 mmol) were added in small portions, at 0 °C. The resulting reaction mixture was allowed to stand at room temperature for the next 16 h. After that, the resulting mixture was diluted with DCM (40 mL) and washed with HCl (2 mol L^{-1}), NaOH (1 mol L^{-1}) and saturated NaCl solution. The resulting crude product was purified by flash column chromatography (*n*-hexane/ethyl acetate, 8:2) to afford title compounds **4a**-**h** in good yields (73–86 %).

Targeted novel *N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)acryloyl)substituted phenyl)benzamide derivatives **4a-h** were synthesized as indicated in Scheme 1. The struc-



Scheme 1

ture of title compounds **4a-h** was ascertained by elemental analysis and spectroscopic methods such as FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry (Tables I and II).

Compounds **4a**-**h** were tested against kinase activities at ten concentrations within the range $1 \times 10^{-4} - 3 \times 10^{-9}$ mol L^{-1} (in 0.4 % DMSO).

Pharmacological activity

Cell culture. – Human osteosarcoma cancer cell lines (MG-63, Saos-2, and U2OS), liver cancer cell line (HepG2), lung cancer cell line (A549), cervical cancer cells (HeLa), normal human lung cells (BEAS-2B), and non-tumorigenic epithelial cell line (MCF 10A) were obtained from ATCC (USA) and cultured as per the instructions. The cells were maintained in a complete growing media (RPMI-1640) (Sigma-Aldrich). All media were supplemented with 10 % of fetal bovine serum (FBS) (PAA, Austria) and 1 % of penicillin-streptomycin (P/S) (Gibco, USA). The control group receives vehicles (0.4 % DMSO).

PI3K enzyme assay. – Inhibition of PI3K activity was determined using ADP-Glo kinase assay from Promega Corporation (USA) (28). Briefly, the test compounds were serially diluted to the desired concentrations and then 1 μL of each was added to a 384-well plate

Table I. Chemical names, molecular formula, elemental analyses, yields and melting points of compounds ***4a–h***

Compd.	Chemical name	Molecular formula	Relative molecular mass (M_r)	Yield (%)[a]	M.p. (°C)	CHN analysis (calcd./found)	R_f value
4a	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)benzamide	$C_{24}H_{17}BrN_4O_2$	473.33	85	214–215	C 60.90; H 3.62; N 11.84 C 60.97; H 3.75; N 11.89	0.79
4b	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-hydroxybenzamide	$C_{24}H_{17}BrN_4O_3$	489.33	75	221–222	C 60.90; H 3.62; N 11.84 C 60.97; H 3.75; N 11.89	0.83
4c	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-methoxybenzamide	$C_{25}H_{19}BrN_4O_3$	503.36	78	245–246	C 59.65; H 3.80; N 11.13 C 59.69; H 3.78; N 11.08	0.71
4d	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-methylbenzamide	$C_{25}H_{19}BrN_4O_3$	487.36	73	229–230	C 59.65; H 3.80; N 11.13 C 59.69; H 3.78; N 11.08	0.62
4e	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-nitrobenzamide	$C_{24}H_{16}BrN_5O_4$	518.33	82	248–249	C 55.61; H 3.11; N 13.51 C 55.68; H 3.23; N 13.54	0.67
4f	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-chlorobenzamide	$C_{24}H_{16}BrClN_4O_2$	507.77	74	256–257	C 56.77; H 3.18; N 11.03 C 56.82; H 3.21; N 11.08	0.72
4g	4-bromo-*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)acryloyl)phenyl)benzamide	$C_{24}H_{16}Br_2N_4O_2$	552.23	84	264–265	C 52.20; H 2.92; N 10.15 C 52.25; H 2.98; N 10.12	0.78
4h	*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-fluorobenzamide	$C_{24}H_{16}BrFN_4O_2$	491.32	86	223–224	C 58.67; H 3.28; N 11.40 C 58.73; H 3.21; N 11.38	0.89

[a] Overall yield.

Table II. Spectral data of compounds ***4a-h***

Compd.	FTIR (KBr, ν_{max}, cm^{-1})	^1H NMR (400 MHz, DMSO-d_6, TMS) (δ, ppm)	^{13}C NMR (100 MHz, DMSO-d_6) (δ, ppm)	MS $(M+H)^+$
4a	3269 (N-H str.), 3145 (triazole ring, C–H str.), 3053 (arom. C-H str.), 1726 (C=O str.), 1658 (CONH str.), 1643 (C=C str.), 1491 (C-Br str.), 1379 (N-N str.), 1082 (C-N str.)	8.41 (s, 1H, triazole-H), 8.03 (d, 2H, *J* = 1.46 Hz, CHx2, Ar-H), 7.86 (d, 2H, *J* = 1.61 Hz, CHx2, Ar-Br), 7.80 (d, 2H, *J* = 1.52 Hz, CHx2, Ar-Br), 7.64 (d, 1H, *J* = 15.46 Hz, CH), 7.58 (d, 2H, *J* = 1.39 Hz, CHx2, Ar-H), 7.55 (t, 1H, *J* = 1.46 Hz, CH, Ar-H), 7.48 (d, 2H, *J* = 0.43 Hz, CHx2, Ar-H), 7.35 (d, 2H, *J* = 0.48 Hz, CHx2, Ar-H), 6.72 (d, 1H, *J* = 15.36 Hz, CH), 4.02 (s, 1H, NH)	189.9, 164.7, 143.8, 135.9, 135.7, 133.6, 134.2, 132.9, 132.1, 131.8, 131.2, 128.9, 128.1, 127.5, 127.2, 123.2, 123.0, 122.1	474.34
4b	3283 (N-H str.), 3229 (O-H str.), 3141 (triazole ring, C–H str.), 3062 (arom. C-H str.), 1708 (C=O str.), 1651 (CONH str.), 1637 (C=C str.), 1495 (C-Br str.), 1384 (N-N str.), 1087 (C-N str.)	9.23 (s, 1H, Ar-OH), 8.49 (s, 1H, triazole-H), 8.02 (d, 2H, *J* = 1.74 Hz, CHx2, Ar-OH), 7.83 (d, 2H, *J* = 1.68 Hz, CHx2, Ar-Br), 7.78 (d, 2H, *J* = 1.48 Hz, CHx2, Ar-Br), 7.68 (d, 1H, *J* = 15.68 Hz, CH), 7.48 (d, 2H, *J* = 1.79 Hz, CHx2, Ar-H), 7.32 (d, 2H, *J* = 1.36 Hz, CHx2, Ar-H), 6.91 (d, 2H, *J* = 1.15 Hz, CHx2, Ar-H), 6.70 (d, 1H, *J* = 15.62 Hz, CH), 3.98 (s, 1H, NH)	189.2, 164.9, 161.5, 143.2, 135.9, 135.4, 133.8, 132.6, 131.5, 131.2, 128.9, 128.3, 127.4, 126.8, 123.2, 123.0, 122.3, 116.9	490.37
4c	3289 (N-H str.), 3148 (triazole ring, C–H str.), 3068 (arom. C-H str.), 2839 (OCH_3 str.), 1714 (C=O str.), 1657 (CONH str.), 1639 (C=C str.), 1498 (C-Br str.), 1385 (N-N str.), 1089 (C-N str.)	8.46 (s, 1H, triazole-H), 8.05 (d, 2H, *J* = 1.78 Hz, CHx2, Ar-OCH_3), 7.83 (d, 2H, *J* = 1.62 Hz, CHx2, Ar-Br), 7.76 (d, 2H, *J* = 1.53 Hz, CHx2, Ar-Br), 7.63 (d, 1H, *J* = 15.62 Hz, CH), 7.44 (d, 2H, *J* = 0.48 Hz,CHx2, Ar-H), 7.35 (d, 2H, *J* = 1.38 Hz, CHx2, Ar-H), 7.03 (d, 2H, *J* = 1.17 Hz, CHx2, Ar-OCH_3), 6.72 (d, 1H, *J* = 15.65 Hz, CH), 3.99 (s, 1H, NH), 3.85 (s, 3H, OCH_3)	190.2, 165.2, 164.1, 143.8, 135.9, 135.6, 133.4, 132.9, 131.9, 131.3, 128.9, 128.2, 127.5, 126.7, 123.3, 123.1, 122.3, 114.5, 55.9	504.36
4d	3292 (N-H str.), 3156 (triazole ring, C–H str.), 3062 (arom. C-H str.), 2962 (CH_3 str.), 1708 (C=O str.), 1651 (CONH str.), 1643 (C=C str.), 1496 (C-Br str.), 1475 (CH_3 bending), 1387 (N-N str.), 1093 (C-N str.)	8.44 (s, 1H, triazole-H), 7.89 (d, 2H, *J* = 1.72 Hz,CHx2, Ar-CH_3), 7.82 (d, 2H, *J* = 1.58 Hz,CHx2, Ar-Br), 7.79 (d, 2H, *J* = 1.49 Hz, CHx2, Ar-Br), 7.65 (d, 1H, *J* = 15.68 Hz, CH), 7.48 (d, 2H, *J* = 1.79 Hz,CHx2, Ar-H), 7.32 (d, 2H, *J* = 1.34 Hz, CHx2, Ar-H), 7.18 (d, 2H, *J* = 1.23 Hz,CHx2, Ar-CH_3), 6.70 (d, 1H, *J* = 15.62 Hz, CH), 3.95 (s, 1H, NH), 2.31 (s, 3H, CH_3)	189.5, 164.9, 143.7, 141.8, 135.9, 135.7, 133.6, 132.9, 131.8, 131.2, 131.0, 129.3, 128.2, 127.5, 127.3, 123.2, 123.0, 122.1, 21.5	488.38

Compd.	FTIR (KBr, ν_{max}, cm^{-1})	^1H NMR (400 MHz, DMSO-d_6, TMS) (δ, ppm)	^{13}C NMR (100 MHz, DMSO-d_6) (δ, ppm)	MS $(M+H)^+$
4e	3294 (N-H str.), 3158 (triazole ring, C–H str.), 3067 (arom. C-H str.), 1714 (C=O str.), 1659 (CONH str.), 1648 (C=C str.), 1539 (NO_2 str.), 1499 (C-Br str.), 1381 (N-N str.), 1095 (C-N str.)	8.42 (s, 1H, triazole-H), 8.14 (d, 2H, *J* = 1.79 Hz, CHx2, Ar-NO_2), 7.91 (d, 2H, *J* = 1.45 Hz,CHx2, Ar-NO_2), 7.81 (d, 2H, *J* = 1.63 Hz, CHx2, Ar-Br), 7.77 (d, 2H, *J* = 1.51 Hz, CHx2, Ar-Br), 7.68 (d, 1H, *J* = 15.62 Hz, CH), 7.51 (d, 2H, *J* = 1.81 Hz, CHx2, Ar-H), 7.36 (d, 2H, *J* = 1.38 Hz, CHx2, Ar-H), 6.73 (d, 1H, *J* = 15.58 Hz, CH), 3.97 (s, 1H, NH)	190.1, 164.9, 151.4, 143.8, 136.9, 135.9, 135.7, 133.7, 132.9, 131.7, 131.4, 129.8, 128.2, 127.2, 124.0, 123.4, 123.1, 122.1	519.35
4f	3296 (N-H str.), 3161 (triazole ring, C–H str.), 3062 (arom. C-H str.), 1719 (C=O str.), 1652 (CONH str.), 1647 (C=C str.), 1493 (C-Br str.), 1389 (N-N str.), 1098 (C-N str.), 846, 796 (C-Cl str.)	8.45 (s, 1H, triazole-H), 7.82 (d, 2H, *J* = 1.68 Hz,CHx2, Ar-Br), 7.80 (d, 2H, *J* = 1.47 Hz, CHx2, Ar-Br), 7.76 (d, 2H, *J* = 1.81 Hz, CHx2, Ar-Cl), 7.64 (d, 1H, *J* = 15.69 Hz, CH), 7.52 (d, 2H, *J* = 1.38 Hz, CHx2, Ar-Cl), 7.46 (d, 2H, *J* = 1.84 Hz, CHx2, Ar-H), 7.33 (d, 2H, *J* = 1.36 Hz, CHx2, Ar-H), 6.70 (d, 1H, *J* = 15.52 Hz, CH), 3.98 (s, 1H, NH)	189.8, 164.9, 143.7, 137.8, 135.8, 135.3, 133.4, 132.9, 132.1, 131.8, 131.3, 130.2, 128.9, 128.2, 127.5, 123.4, 123.0, 122.1	508.78
4g	3299 (N-H str.), 3157 (triazole ring, C–H str.), 3061 (arom. C-H str.), 1723 (C=O str.), 1658 (CONH str.), 1642 (C=C str.), 1498 (C-Br str.), 1393 (N-N str.), 1087 (C-N str.)	8.43 (s, 1H, triazole-H), 7.95 (d, 2H, *J* = 1.78 Hz,CHx2, Ar-Br), 7.82 (d, 2H, *J* = 1.62 Hz, CHx2, Ar-Br), 7.79 (d, 2H, *J* = 1.47 Hz, CHx2, Ar-Br), 7.69 (d, 2H, *J* = 1.34 Hz, CHx2, Ar-Br), 7.63 (d, 1H, *J* = 15.61 Hz, CH), 7.49 (d, 2H, *J* = 1.81 Hz, CHx2, Ar-H), 7.36 (d, 2H, *J* = 1.32 Hz, CHx2, Ar-H), 6.73 (d, 1H, *J* = 15.59 Hz, CH), 3.99 (s, 1H, NH)	189.8, 165.2, 143.9, 135.9, 135.7, 133.4, 133.1, 132.9, 131.9, 131.5, 131.2, 129.8, 128.0, 127.6, 126.8, 123.4, 123.1, 122.4	553.23
4h	3295 (N-H str.), 3163 (triazole ring, C–H str.), 3058 (arom. C-H str.), 1718 (C=O str.), 1668 (CONH str.), 1649 (C=C str.), 1495 (C-Br str.), 1391 (N-N str.), 1168 (C-F str.), 1082 (C-N str.)	8.42 (s, 1H, triazole-H), 7.97 (d, 2H, *J* = 1.73 Hz,CHx2, Ar-F), 7.81 (d, 2H, *J* = 1.60 Hz, CHx2, Ar-Br), 7.80 (d, 2H, *J* = 1.54 Hz, CHx2, Ar-Br), 7.67 (d, 1H, *J* = 15.69 Hz, CH), 7.46 (d, 2H, *J* = 1.80 Hz, CHx2, Ar-H), 7.42 (d, 2H, *J* = 1.08 Hz, CHx2, Ar-Br), 7.32 (d, 2H, *J* = 1.33 Hz, CHx2, Ar-H), 6.71 (d, 1H, *J* = 15.49 Hz, CH), 3.98 (s, 1H, NH)	189.9, 166.5, 164.7, 143.8, 135.9, 135.7, 133.5, 132.9, 131.7, 131.2, 129.7, 129.1, 128.1, 127.4, 123.2, 123.0, 122.1, 115.8	492.32

arom. – aromatic, str. – stretching

(Corning, USA) as an assay plate. Four microliters of PIP2:3PS lipid kinase substrate working solution was added to each well, followed by 4 μL of kinase, except for the control well, where 4 μL of kinase reaction buffer was added. To this, 5 μL of ADP-Glo™ reagent (with 10 mmol L^{-1} $MgCl_2$) were added and incubated at room temperature for 40 min. Afterwards, 10 μL of kinase detection reagent were added and incubated at room temperature for 30 min. The mixture was shaken for 1 min and equilibrated for 40 min before the reading of luminescence on a plate reader was taken. Finally, conversion data was collected on Flex station and RLU (relative light unit) values were converted to inhibition values as per the kit protocol. All of the compounds were tested three times, and the results are expressed as IC_{50}.

mTOR enzyme assay. – The mTOR kinase activity of all the compounds was determined using LANCE® ultra time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Invitrogen, USA), following the manufacturer's instructions (29). Briefly, mTOR enzyme (0.1 μg mL^{-1}), ATP (3 μmol L^{-1}), GFP-4EBP1 peptide (0.4 μmol L^{-1}) and test compounds were diluted in kinase buffer. The reactions were performed in black 384-well proxiplates (Corning, USA) at room temperature for 1 h and stopped by adding EDTA (10 mmol L^{-1}). Tb-antiphospho--4EBP1 (Thr37/46) antibody (PerkinElmer) was then added to each well to a final concentration of 2 nmol L^{-1}, and the mixture was incubated at room temperature for 30 min. The intensity of the light emission was measured with Spectramax 190 reader (Molecular Devices, USA) in TR-FRET mode (excitation at 320 nm and emission at 665 nm). All of the compounds were tested three times, and the results are expressed as IC_{50}.

Cell proliferation assay on various cancer cells. – Cell proliferation was determined by MTT assay as per the adapted procedure (6). Briefly, the cells were harvested, counted and seeded at 1×10^4 cells per well in the 96-well plate for 24 hours. The following day, cells were treated with various concentrations of compounds **4e**, **4f**, **4g** and gedatolisib ranging from 100 to 1.25 μg mL^{-1} in 0.4 % DMSO, and incubated for 48 hours at 37 °C with 5 % CO_2. After the medium was aspirated, 20 μL of 5 mg mL^{-1} MTT reagent (Merck, USA) was added to each well and incubated for 3 hours. The excessive liquid was gently removed and 100 μL of DMSO (0.4 %) were added to the wells. Then, the plate was read at 575 nm by using the microtiter plate reader (μQuant, Bio-Tek Instrument, USA). The experiment was performed in triplicate, and cell survival was calculated as the percentage of MTT-formation inhibition.

Annexin V/PI assay. – The apoptosis in Saos-2 cells was determined using an Annexin V fluorescence kit (BD Pharminogen, USA) as per the earlier reported procedure without any modifications (30). Briefly, cells were seeded for 24 hours at a density of 1.8×10^5 cells per well in a 6-well plate. Then, the seeded cells were treated with diverse concentrations of compound **4e** (2.5, 5 and 10 μmol L^{-1}) and gedatolisib (2.5 μmol L^{-1}) in 0.4 % DMSO. After 48 hours the treated cells were harvested and collected as a pellet. Next, the pellet was re-suspended in a 400 μL binding buffer, and stained with 5.0 μL of FITC-Annexin V and 5.0 μL of PI provided in the kit. Afterwards, the cells were analyzed by BD FACS Calibur (Becton Dickinson, USA). The results were analyzed using CellQuest 3.3 software. The experiments were performed in triplicate.

Statistical analysis

The data reported as mean ± SEM of three different individual experiments were examined by one-way analysis of variance using GraphPad Prism 5.0 (GraphPad Software). $p < 0.05$ indicates statistical significance.

RESULTS AND DISCUSSION

Synthesis of target molecules

Synthesis of compounds **1**, **2** and **3** was performed as previously described (26), the detailed information on these syntheses are provided in short in the supplementary material (Tables SI and SII). Scheme 1 displays the whole synthetic pathway including targeted novel *N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)acryloyl)substituted phenyl)benzamide derivatives **4a-h**. They were prepared by reaction of compound **3** with the mentioned substituted benzoyl chlorides in dichloromethane by straight acylation. Physico-chemical and spectral data for compounds **4a-h** are given in Tables I and II.

Kinase inhibitory activity

The designed compounds were tested against PI3K and mTOR and the comparative inhibitory activity has been provided in Table III. It has been found that compounds **4a-h** showed significant to moderate IC_{50} values against both kinases in the nanomolar range. Compound **4a** with no substitution showed the lowest inhibitory activity against PI3K (IC_{50} = 67.78 ± 2.60 nmol L^{-1}) with moderate inhibitory activity against mTOR kinase (IC_{50} = 59.27 ± 2.73 nmol L^{-1}). However, upon substitution with a hydroxyl group (**4b**), activity significantly improved against PI3K with a slight reduction in mTOR kinase activity. The inhibitory activity was found to be significantly improved against both the tested kinases upon replacing *p*-hydroxy with *p*-methyl in the case of compound **4c**. The presence of the *p*-methoxy group in **4d** was found detrimental for the activity and rendered the compound moderately active against both PI3K and mTOR. Interestingly, the introduction of electron-withdrawing groups as substituents, *i.e.*, in compounds **4e**, **4f**, **4g** and **4h** showed a significant increase in inhibitory activity against the tested kinases (PI3K and mTOR). The presence of the *p*-nitro group in **4h** showed the least inhibitory activity among the tested molecules. Upon replacing the nitro group with bromine (**4g**) the activity markedly improved, and it further increased in the case of **4f** (*p*-chloro) and **4e** (*p*-fluoro). The comparison of inhibitory

*Table III. Inhibitory activity of compounds **4a-h** against PI3K and mTOR kinase*

Compd.	Substituent	Kinase inhibition (IC_{50}, nmol L^{-1})[a]	
		PI3K	mTOR
4a	H	67.78 ± 2.60	59.27 ± 2.73
4b	OH	56.82 ± 2.31	61.43 ± 2.45
4c	CH_3	39.30 ± 1.32	27.74 ± 1.42
4d	OCH_3	45.67 ± 1.34	40.31 ± 1.05
4e	F	4.23 ± 0.32	2.30 ± 0.15
4f	Cl	10.04 ± 0.56	13.63 ± 0.62
4g	Br	19.34 ± 0.27	27.04 ± 0.45
4h	NO_2	32.26 ± 0.43	30.40 ± 0.56
Gedatolisib (standard)		6.04 ± 0.45	2.50 ± 0.23

[a] Mean ± SEM of three replicates.

activity suggests that none of the tested compounds showed higher activity than gedatolisib (a standard inhibitor of tested kinases) against both PI3K (IC_{50} = 6.04 ± 0.45 nmol L^{-1}) and mTOR (IC_{50} = 2.5 ± 0.23 nmol L^{-1}), except compound **4e** with IC_{50} of 4.23 ± 0.32 nmol L^{-1} and 2.3 ± 0.15 nmol L^{-1} against PI3K and mTOR, resp. Compound **4e** was identified as the most potent inhibitor of PI3K and mTOR among the tested series with activity comparable to that of gedatolisib, while non-substituted derivatives were found least active.

The structure-activity relationship study suggests that compounds having an electron-withdrawing group showed a better inhibitory profile as compared to their electron-donating derivatives. The steric size, lipophilicity, molecular volume and inductive effect of the electron-withdrawing group in *p*-position plays an important role in the activity against tested kinases.

*Anticancer activity of compounds **4e-g***

Based on the good results in kinase inhibitory assays, the top-three ranked compounds (**4e-g**) were subjected to further examinations of anticancer activity against diverse cancer cell lines, such as human OS cancer cell line (MG-63), liver cancer cell line (HepG2), lung cancer cell line (A549) and cervical cancer (HeLa) and the results are displayed in Table IV. The effects of these derivatives were also defined on the cells viability experiments and the results have been presented in Supplementary materials (Fig. S1). The compounds showed potent to moderate activity against all the tested cell lines with significant inhibition of the cellular viability which was found to be in agreement with the MTT assay. Compound **4g** showed the lowest activity among all the tested derivatives, against all cell lines, except Hep G2. It showed the lowest activity against the A549 with significant activity against the MG-63 cells. The activity was shown to considerably increase in the case of compound **4f**, except against Hep G2 cells, where it displayed the lowest activity. The most significant anticancer activity was reported for compound **4e** against all cell lines with maximum potency against MG-63 cells, namely, Saos-2 cells. These results were also found in line with cell viability experiments (Fig. S2, Supplementary materials). However, none of the compounds was found to be toxic to normal cells (normal human lung cells BEAS-2B and non-tumorigenic epithelial cell line MCF 10A) at the

*Table IV. Inhibitory activity of compounds **4e-g** against cancer and normal cells*

Compd.	IC_{50} (μmol L^{-1})[a]							
	HeLa	MG-63	Hep G2 (liver)	A549 (lung)	Osteocarcinoma cells		Normal cells	
					U2OS	Saos-2	BEAS-2B	MCF 10A
4e	8.12 ± 0.67	1.03 ± 0.23	16.72 ± 1.67	17.78 ± 2.43	0.45 ± 0.02	0.23 ± 0.02	> 200	> 200
4f	12.34 ± 1.05	5.62 ± 0.94	45.53 ± 2.10	38.42 ± 2.71	–	–	> 200	> 200
4g	34.56 ± 1.39	7.34 ± 1.43	37.75 ± 2.83	54.62 ± 3.53	–	–	> 200	> 200
Gedatolisib (standard)	5.32 ± 0.25	0.25 ± 0.76	11.37 ± 1.56	9.05 ± 2.78	0.53 ± 0.04	0.15 ± 0.01	> 200	> 200

[a] Mean ± SEM of three replicates.

highest tested dose of 200 μmol L^{-1}. Upon closely observing Table IV, gedatolisib was found somewhat more active than tested compounds **4e**, **4f** and **4g** against HeLa, MG-63, A549 and Hep G2 cells.

*Anti-osteosarcoma activity of compound **4e***

As shown in Table IV, compound **4e** potently inhibits OS cells like Saos-2, with the least activity against MG-63 cells. Significant inhibitory activity was reported against U2OS cells. In terms of IC_{50} values, comparable activity of **4e** against U2OS and Saos-2 cells to that of gedatolisib is evidenced.

As shown in Fig. 1 compound **4e** induces the apoptosis of Saos-2 cells in a dose-dependent manner; the greatest apoptotic activity was reported at the highest tested dose of 10 μmol L^{-1}. Moreover, the standard gedatolisib showed the highest apoptotic activity at the dose of 2.5 μmol L^{-1}.

In the previous studies, various 1,2,3-triazole-chalcone derivatives were synthesized and found to exhibit significant anticancer activity (31, 32). Fu and co-authors developed a

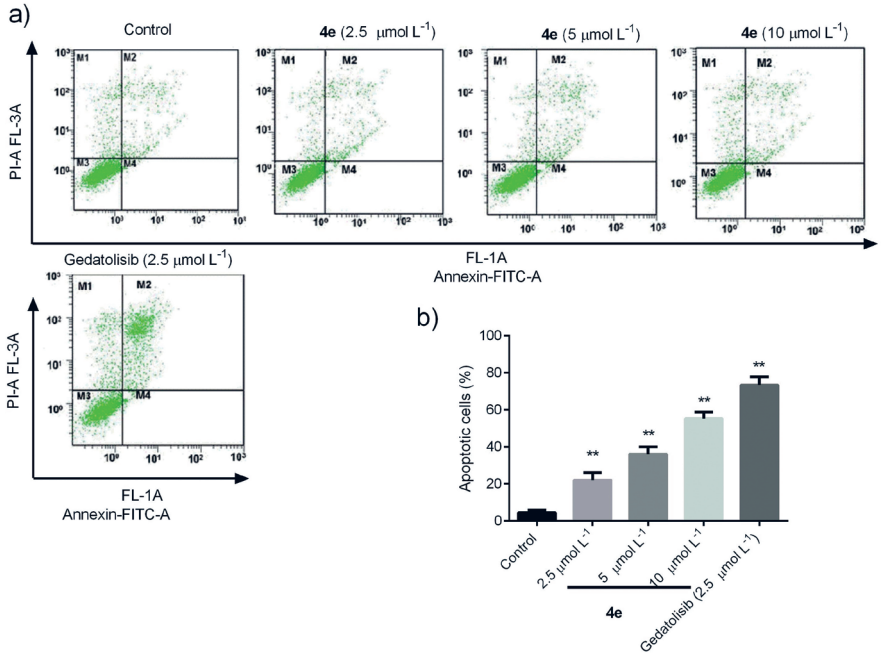


Fig. 1. Effect of compound **4e** and gedatosilib on apoptosis of Saos-2 cells. a) Flow cytometry: M1 – necrotic cells, M2 – late and M4 – early apoptotic cells, M3 – viable cells. Scatter plots are given as a function of fluorescence intensity of Annexin V-FITC *vs.* PI, where X-axis: number of cells positive for Annexin V-FITC staining, Y-axis: number of propidium iodide-stained cells. [Emission filters: filter 530/30 (detector FL-1A) to read Annexin V-fluorescein (FITC channel), and filter 670 LP (detector FL-3A) to read propidium iodide]. b) Percent of Saos-2 apoptotic cells. Values represent the mean ± SD, $n = 3$. Statistical significance: $^{**}p < 0.01$ *vs.* the control group.

series of 1,2,3-triazole-chalcone hybrids and found that these molecules showed considerable anticancer activity against SK-N-SH, HepG2 and MGC-803 cells (32). These compounds caused induction of apoptosis and G1 phase cell cycle arrest, particularly against SK-N-SH cancer cells. The same group further developed a series of novel chalcone-1,2,3--triazole-azole derivates and found that these molecules showed marked inhibitory activity against SK-N-SH cancer cells by inducing apoptosis (33). Our results were found to be in agreement with the above studies where the anticancer activity of 1,2,3-triazole-chalcone hybrids was attributed mainly to induction of apoptosis. However, our study lacks a more detailed mechanistic analysis of the most potent inhibitor **4e** against OS cell, like Western blot analysis to study the effect of the designed compound on the downstream mediator and phosphorylation of PI3K/Akt/mTOR signalling pathway, cell-cycle analysis and *in vivo* study.

CONCLUSIONS

This study presents the discovery of novel 1,2,3-triazole chalcone derivatives as potential anti-osteosarcoma agents *via* inhibition of the PI3K/Akt/mTOR signalling pathway. Among the tested series, compound **4e** (*N*-(4-(3-(1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl) acryloyl)phenyl)-4-nitrobenzamide), showed a better inhibitory profile than gedatolisib against PI3K, while being approximately comparable to gedatolisib against mTOR. However, its inhibitory activity was somewhat lower to comparable against all the tested cancer cell lines as compared to gedatolisib, except for U2OS and Saos-2 cells, where it was found approximately equipotent as gedatolisib. It further significantly induced apoptosis in Saos-2 cells in a dose-dependent manner and was found non-toxic to normal cell lines. However, there are still some limitations of the study. First, this study lacks *in vivo* biological experiments to confirm the anti-osteosarcoma activity of compound **4e**. Secondly, a detailed mechanistic analysis of the anticancer effect of compound **4e** against osteosarcoma is still worth being carried out.

Supplementary materials are available upon request.

Conflict of interest. – Authors declare no conflict of interest

Author's contributions. – QS performed experiments, analyzed the data, BX performed the experiment, ZT performed the experiment and analyzed the data, ZG conceptualized and supervised the study. All authors approved the current version of the manuscript.

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