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ORIGINAL ARTICLE

Synthesis and anticancer activity of novel 3,6-disubstituted 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole derivatives

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Abstract The development of new antitumor agents is one of the most pressing research areas in medicinal chemistry and medicine. The importance of triazole and thiadiazole rings as scaffolds present in a wide range of therapeutic agents has been well reported and has driven the synthesis of a large number of novel antitumor agents. The presence of these heterocycles furnishes extensive synthetic possibilities due to the presence of several reaction sites. Prompted by these data we designed, synthesized and evaluated a series of novel 3,6-disubstituted 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole derivatives as potential anticancer agents. We emphasized in the strategy of combining two chemically different but pharmacologically compatible molecules (the 1,2,4-triazole and 1,3,4-thiadiazole) in one frame. Several of the newly synthesized 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole derivatives showed substantial cytostatic and cytotoxic antineoplastic activity *in vitro*, while they have produced relatively low acute toxicities *in vivo*, giving potentially high therapeutic ratios. *In silico* screening has revealed several protein targets including apoptotic protease-activating factor 1

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(APAF1) and tyrosine-protein kinase HCK which may be involved in the biological activities of active analogues.

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1. Introduction

Cancer is among the leading causes of morbidity and mortality worldwide and remains a major public health issue at the beginning of the 21st century. Approximately 14 million new cases and 8.2 million cancer related deaths were reported in 2012. More than 60% of global new annual cases occur in Africa, Asia and Central and South America. These developing and under-developed regions account for 70% of the world's cancer deaths. The number of new cases is expected to rise by about 70% over the next 2 decades (World Cancer Report, 2014).

The successful treatment of cancer remains a significant challenge because of the general toxicity associated with the clinical use of traditional cancer chemotherapeutic agents. Significant side effects such as nausea, vomiting, diarrhea, hair loss and serious infections (mostly due to leukopenia) often accompany chemotherapy. Therefore, the need for accelerated development of new, more effective as well as less toxic chemotherapeutic agents is unquestioned. The development of new antitumor agents is one of the most urgent research areas in medicinal chemistry and oncology.

The importance of triazole or thiadiazole rings as scaffolds present in a range of therapeutic agents, which furnish extensive synthetic possibilities due to the presence of several reaction sites, has driven the synthesis of a large number of novel antitumor agents bearing these heterocycles. Thiadiazoles have been of great interest as core structures of antitumor agents for many years (Hill, 1980; Nelson et al., 1977; Tsukamoto et al., 1975), possibly due to the presence of toxophoric N-C-S moiety (Omar and Aboulwafa, 1986). Also, the triazole ring is highly reactive, due to the presence of an acidic proton at C-2, and emerges as an important synthon to generate new chemical entities. Diverse modifications of the triazole or thiadiazole rings at various positions have led to a variety of novel compounds with wide spectrum of pharmacological activities.

As a result of the above findings, several patents were registered from 2008 to present concerning new triazole and thiadiazole ring containing derivatives useful for the development of new anticancer drug molecules (Morigi et al., 2015).

Therefore, the fused compounds and their derivatives obtained by fusing the bio-labile 1,2,4-triazole and 1,3,4-thiadiazole rings together represent an interesting class of heterocyclic compounds with a broad spectrum of pharmacological activities which include antifungal (Chaturvedi et al., 1988; Karabasanagouda et al., 2007), antibacterial (Demirbas et al., 2005; Holla et al., 1996; Zhang et al., 1997), antiviral (Invidiata et al., 1996; Kritsanida et al., 2002; Srivastava et al., 1994), anti-inflammatory (Amir et al., 2008; Chawla et al., 2012; Prasad et al., 1986), analgesic (Chawla et al., 2012; Srivastava et al., 1994), and anthelmintic (El-Khawass et al., 1989; Hussain and Kumar, 1992) while of particular interest is the impressive anticancer/antitumor activity (Al-Masoudi and Al-Soud, 2008; Chowrasia et al., 2013; Ibrahim, 2009; Kaliappan and Parthiban, 2010). More specifically, the cytotoxic potency of 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazoles against various cancer cell lines has been extensively reported (Husain et al., 2013; Shivarama Holla et al., 2002; Subrahmanya Bhat et al., 2004; Sunil et al., 2010).

Prompted by these observations and in continuation of our search for alternate chemotherapeutic compounds, we synthesized and evaluated a series of novel 3,6-disubstituted 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole derivatives as potential anticancer agents. In particular, we emphasized the strategy of combining two chemically different but pharmacologically compatible molecules (the 1,2,4-triazole and 1,3,4-thiadiazole) in one frame.

A simple sulfonamide may play the role of the initial lead compound for the synthesis of the target triazolo thiadiazoles. In general, a novel series of sulfonamide derivatives containing different, biologically active, moieties including pyridine, thiophene or benzothiophene moieties have also shown promising anticancer activity. The chemical motif of aromatic/heterocyclic sulfonamide has been correlated with a variety of antitumor mechanisms, such as carbonic anhydrase inhibition, cell cycle arrest in the G1 phase, disruption of microtubule assembly, functional suppression of the transcriptional activator NF- κ B, and angiogenesis (matrix metalloproteinase, MMP) inhibition among others (Ghorab et al., 2015).

2. Materials and methods

2.1. Chemistry

The 2-(N,N-dimethylsulfamoyl)-4,5-dimethoxy-phenylacetylhydrazide (I), prepared from ethyl-2-(N,N-dimethylsulfamoyl)-4,5-dimethoxy-phenylacetate by treatment with hydrazine hydrate in xylol (Ezabadi et al., 2008), was allowed to react with carbon disulfide in the presence of potassium hydroxide in ethanol to afford the corresponding intermediate potassium thiocarbamate (II). This salt underwent ring closure with excess of hydrazine hydrate to give 5-[2-(N,N-dimethylsulfamoyl)-4,5-dimethoxybenzyl]-3-mercapto-4-amino-1,2,4-triazole (III). The resulted triazole was further converted to 3,6-disubstituted 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazoles (IV) by condensing with various aromatic acids in the presence of phosphorus oxychloride as outlined in Scheme 1 and Table 1.

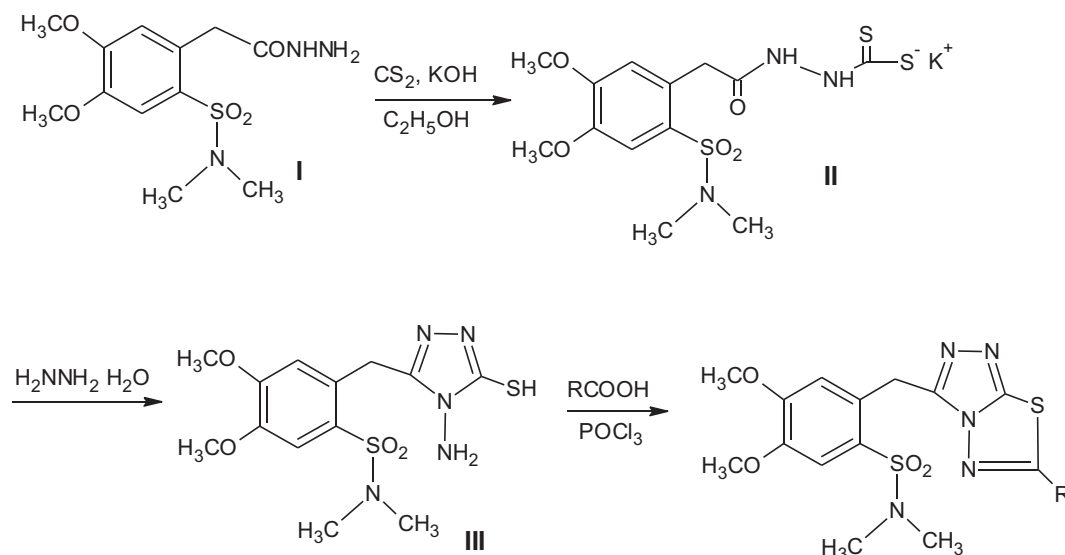
2.1.1. 5-[2-(N,N-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-4-amino-3-mercapto-1,2,4-triazole (III)

To a cold stirred solution of 2-(N,N-dimethylsulfamoyl)-4,5-dimethoxy-phenylacetyl hydrazide (0.01 mol) in absolute ethanol (150 mL) containing potassium hydroxide (0.015 mol), carbon disulfide (0.015 mol) was added gradually. The reaction mixture was stirred at room temperature for 20 h where upon a precipitate of the corresponding potassium dithiocarbamate was separated. Dry ether (150 mL) was then added to complete the precipitation of the formed salt. The obtaining product was filtered, washed with dry ether and dried.

The above salt was suspended in 80% hydrazine hydrate (0.02 mol), stirred and heated under reflux for 2 h. The reaction mixture was cooled, diluted with ice cold water and neutralized with 10% hydrochloric acid. The precipitate obtained, was filtered, washed thoroughly with cold water, dried and recrystallized from methanol.

Yield: 56%, M.p. 194–196 °C (CH₃OH). I.R. ν cm⁻¹ 3314, 3250 (NH), 2942 (CH), 1604 (C=N), 1574, 1510, 1448 (C=C), 1265 (N=N=C), 1326 (S—O anti sym), 1135 (S—O sym).

¹H NMR δ (ppm): 13.39 (s, 1H, SH), 7.25 (s, 1H, ArH), 7.02 (s, 1H, ArH), 5.56 (s, 2H, NH₂), 4.30 (s, 2H, CH₂), 3.79 (s, 6H, 2CH₃—O—), 2.59 (s, 6H, N(CH₃)₂).



Scheme 1 Synthetic route for the newly synthesized analogues.

Table 1 The 20 novel triazolo-thiadiazole analogues.

Analogue	Substituent	Analogue	Substituent
IVa	C ₆ H ₅ —	IVk	4-CH ₃ O—C ₆ H ₄ CH ₂ —
IVb	4-Cl—C ₆ H ₄ —	IVl	3,4-CH ₃ O—C ₆ H ₃ CH ₂ —
IVc	2-NH ₂ —C ₆ H ₄ —	IVm	C ₆ H ₅ —OCH ₂ —
IVd	3-NH ₂ —C ₆ H ₄ —	IVn	C ₆ H ₅ —CH=CH
IVe	4-NH ₂ —C ₆ H ₄ —	IVo	2-CH ₃ O—C ₆ H ₄ CH ₂ CH ₂ —
IVf	2-Cl—4-NO ₂ —C ₆ H ₃ —	IVp	4-CH ₃ O—C ₆ H ₄ CH ₂ CH ₂ —
IVg	3-CH ₃ —4-NO ₂ —C ₆ H ₃ —	IVq	C ₆ H ₅ CH ₂ CH ₂ CH ₂ —
IVh	3,4,5-CH ₃ O—C ₆ H ₂ —	IVr	
IVi	C ₆ H ₅ CH ₂ —	IVs	
IVj	3-CH ₃ O—C ₆ H ₄ CH ₂ —	IVt	

Analysis: C₁₃H₁₉N₅O₄S₂ (373). Calc. %: C:41.82, H:5.09, N:18.76. Found: C:41.77, H:5.12, N:18.79.

2.1.2. General procedure for the synthesis of 3,6-disubstituted-1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles (IVa-t)

An equimolar mixture of triazole (III) (0.01 mol) and appropriate aromatic acids (0.01 mol) in dry phosphorus oxychloride (5 mL) was refluxed for 2 h. The reaction mixture was cooled to room temperature and then gradually poured onto crushed ice with stirring. Finally, to remove the excess of phosphorus oxychloride, powdered potassium carbonate and the required amount of solid potassium hydroxide were added till the pH of the mixture was raised to 8. The solid was collected

by filtration, washed thoroughly with cold water, dried and recrystallized from the appropriate solvent.

The following compounds were prepared by an analogous procedure.

2.1.2.1. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-phenyl-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (IVa). Yield: 74%, M.p. 212–214 °C (CH₃OH), ¹H NMR (CDCl₃) δ 7.95 (d, *J* = 6.9 Hz, 2H), 7.68 (m, 1H), 7.63 (m, 2H), 7.30 (s, 1H), 7.21 (s, 1H), 4.78 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.63 (s, 6H), I.R. ν cm⁻¹ 1601 (C=N), 1573, 1513, 1470, 1446 (C=C), 1265 (N=N=C), 1328 (S=O antisym.), 1141 (S=O sym.), Analysis: C₂₀H₂₁N₅O₄S₂ (459). Calc. %: C:52.28, H:4.57, N:15.25. Found: C:52.25, H:4.53, N:15.27.

2.1.2.2. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(4-chloro-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVb**). M.p. 228–229 °C (CH₃OH–CH₂Cl₂), ¹H NMR (CDCl₃) δ 7.97 (d, *J* = 8.5 Hz, 2H), 7.71 (d, *J* = 8.6 Hz, 2H), 7.30 (s, 1H), 7.20 (s, 1H), 4.78 (s, 2H), 3.84 (s, 3H), 3.79 (s, 3H), 2.62 (s, 6H), I.R. ν cm⁻¹ 1600(C=N), 1573, 1519, 1470 (C=C), 1272 (N–N=C), 1339(S–O antisym.), 1138 (S–O sym), Analysis: C₂₀H₂₀N₅O₄S₂Cl (493.5). Calc. %: C:48.63, H:4.05, N:14.18. Found: C:48.65, H:4.01, N:14.21.

2.1.2.3. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(2-amino-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVc**). Yield: 36%. M.p. 219–220 °C (C₂H₅OH), ¹H NMR (CDCl₃) δ 7.30 (s, 1H), 7.23 (t, *J* = 7.8 Hz, 1H), 7.20 (s, 1H), 7.13 (m, 1H), 7.03 (d, *J* = 8.35 Hz, 1H), 6.82 (d, *J* = 8.2 Hz, 1H), 4.75 (s, 2H), 3.84 (s, 3H), 3.79 (s, 3H), 2.61 (s, 6H), I.R. ν cm⁻¹ 2436 3382, 3267 (N–H), 1608 (C=N), 1555, 1514, 1474 (C=C), 1265 (N–N=C), 1328 (S–O antisym.), 1141 (S–O sym.), Analysis: C₂₀H₂₂N₆O₄S₂ (474). Calc. %: C:50.63, H:4.64, N:17.72. Found: C:50.60, H:4.61, N:17.75.

2.1.2.4. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3-amino-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVd**). Yield: 20%. M.p. 164–165 °C (C₂H₅OH), ¹H NMR (CDCl₃) δ 7.44 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.29 (m, 1H), 7.28 (s, 1H), 7.16 (s, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 6.71 (bs, 2H, NH₂), 6.68 (t, *J* = 7.5 Hz, 1H), 4.81 (s, 2H), 3.84 (s, 3H), 3.78 (s, 3H), 2.63 (s, 6H), I.R. ν cm⁻¹, 3450, 3347 (N–H), 1626 (C=N), 1579, 1512, 1473 (C=C), 1272 (N–N=C), 1322 (S–O antisym.), 1129 (S–O sym.), Analysis: C₂₀H₂₆N₆O₄S₂ (474). Calc. %: C:50.63, H:4.64, N:17.72. Found: C:50.65, H:4.67, N:17.69.

2.1.2.5. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(4-amino-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVe**). Yield: 18%. M.p. 179–181 °C (C₂H₅OH), ¹H NMR (CDCl₃) δ 7.58 (dd, *J* = 8.6, 2.9 Hz, 2H), 7.29 (d, *J* = 3.0 Hz, 1H), 7.19 (d, *J* = 2.8 Hz, 1H), 6.68 (dd, *J* = 8.6, 2.9 Hz, 2H), 6.17 (s, 2H), 4.72 (s, 2H), 3.84 (s, 3H), 3.78 (s, 3H), 2.61 (s, 6H), I.R. ν cm⁻¹ 3457, 3348, 3237 (N–H), 1603 (C=N), 1577, 1518, 1461 (C=C), 1265 (N–N=C), 1331 (S–O antisym.), 1138 (S–O sym.), Analysis: C₂₀H₂₆N₆O₄S₂ (474). Calc. %: C:50.63, H:4.64, N:17.72. Found: C:50.60, H:4.59, N:17.70.

2.1.2.6. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(2-chloro-4-nitro-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVf**). Yield: 65%. M.p. 216–217 °C (CH₃OH), ¹H NMR (CDCl₃) δ 8.57 (d, *J* = 2.3 Hz, 1H), 8.40 (dd, *J* = 8.7, 2.3 Hz, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 7.30 (s, 1H), 7.20 (s, 1H), 4.80 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.61 (s, 6H), I.R. ν cm⁻¹ 1603(C=N), 1573, 1518, 1476 (C=C), 1269 (N–N=C), 1331 (S–O antisym.), 1138 (S–O sym.), Analysis: C₂₀H₁₉N₆O₆S₂Cl (538.5). Calc. %: C:44.56, H:3.52, N:15.60. Found: C:44.53, H:3.55, N:15.63.

2.1.2.7. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3-methyl-4-nitro-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVg**). Yield: 46%. M.p. 142–144 °C (CH₃OH), ¹H NMR (CDCl₃) δ 8.18 (d, *J* = 8.5 Hz, 1H), 8.08 (s, 1H), 8.02 (d,

J = 8.5 Hz, 1H), 7.30 (s, 1H), 7.22 (s, 1H), 4.80 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.62 (s, 6H), I.R. ν cm⁻¹ 1600(C=N), 1573, 1510, 1476 (C=C), 1271 (N–N=C), 1344 (S–O antisym.), 1133 (S–O sym.), Analysis: C₂₁H₂₂N₆O₆S₂ (518). Calc. %: C:48.64, H:4.24, N:16.21. Found: C:48.68, H:4.21, N:16.25.

2.1.2.8. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3,4,5-trimethoxy-phenyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVh**). Yield: 38%. M.p. 169–170 °C (CH₃OH), ¹H NMR (CDCl₃) δ 7.30 (s, 1H), 7.22 (s, 1H), 7.14 (s, 2H), 4.78 (s, 2H), 3.89 (s, 6H), 3.84 (s, 3H), 3.80 (s, 3H), 3.76 (s, 3H), 2.62 (s, 6H), I.R. ν cm⁻¹ 1630(C=N), 1586, 1459, 1414 (C=C), 1267 (N–N=C), 1333 (S–O antisym.), 1127 (S–O sym.), Analysis: C₂₃H₂₇N₅O₇S₂ (549). Calc. %: C:50.27, H:4.92, N:12.75. Found: C:50.25, H:4.96, N:12.78.

2.1.2.9. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-benzyl-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVi**). Yield: 18%. M.p. 178–179 °C (CH₃OH); ¹H NMR (CDCl₃) δ 7.42–7.36 (m, 4H), 7.36–7.30 (m, 1H), 7.28 (s, 1H), 7.13 (s, 1H), 4.69 (s, 2H), 4.44 (s, 2H), 3.84 (s, 3H), 3.76 (s, 3H), 2.57 (s, 6H), I.R. ν cm⁻¹ 1601 (C=N), 1565, 1517, 1475 (C=C), 1267 (N–N=C), 1339 (S–O antisym.), 1140 (S–O sym.), Analysis: C₂₁H₂₃N₅O₄S₂ (473). Calc. %: C:53.27, H:4.86, N:14.80. Found: C:53.23, H:4.89, N:14.83.

2.1.2.10. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3-methoxy-benzyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVj**). Yield: 58%. M.p. 165–166 °C (CH₃OH), ¹H NMR (CDCl₃) δ 7.30 (d, *J* = 8.0 Hz, 1H), 7.29 (s, 1H), 7.13 (s, 1H), 6.97 (s, 1H), 6.94 (d, *J* = 7.7 Hz, 1H), 6.90 (dd, *J* = 8.3, 2.5 Hz, 1H), 4.70 (s, 2H), 4.41 (s, 2H), 3.84 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H), 2.57 (s, 6H), I.R. ν cm⁻¹ 1607 (C=N), 1581, 1515, 1491 (C=C), 1266 (N–N=C), 1334 (S–O antisym.), 1139 (S–O sym.), Analysis: C₂₂H₂₅N₅O₅S₂ (503). Calc. %: C:52.48, H:4.97, N:13.91. Found: C:52.44, H:4.95, N:12.88.

2.1.2.11. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(4-methoxy-benzyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVk**). Yield: 52%. M.p. 184–185 °C (CH₃OH), ¹H NMR (CDCl₃) δ 7.30 (d, *J* = 8.6 Hz, 2H), 7.29 (s, 1H), 7.13 (s, 1H), 6.94 (d, *J* = 8.6 Hz, 2H), 4.69 (s, 2H), 4.36 (s, 2H), 3.84 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H), 2.57 (s, 6H), I.R. ν cm⁻¹ 1610 (C=N), 1571, 1514, 1476 (C=C), 1267 (N–N=C), 1340 (S–O antisym.), 1140 (S–O sym.), Analysis: C₂₂H₂₅N₅O₅S₂ (503). Calc. %: C:52.48, H:4.97, N:13.91. Found: C:52.45, H:4.99, N:13.94.

2.1.2.12. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3,4-dimethoxy-benzyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVl**). Yield: 50%. M.p. 139–140 °C (CH₃OH), ¹H NMR (CDCl₃) δ 7.29 (s, 1H), 7.13 (s, 1H), 6.99 (s, 1H), 6.95 (d, *J* = 8.2 Hz, 1H), 6.90 (dd, *J* = 8.3, 2.0 Hz, 1H), 4.70 (s, 2H), 4.35 (s, 2H), 3.84 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H), 2.58 (s, 6H), I.R. ν cm⁻¹ 1602 (C=N), 1555, 1516, 1462 (C=C), 1266 (N–N=C), 1336 (S–O antisym.), 1139 (S–O sym.), Analysis: C₂₃H₂₇N₅O₆S₂ (533). Calc. %: C:51.78, H:5.06, N:13.13. Found: C:51.81, H:5.10, N:13.15.

2.1.2.13. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-phenoxy-methyl-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVm**). Yield: 41%. M.p. 178–179 °C (CH₃OH–CH₂Cl₂), ¹H NMR (CDCl₃) δ 7.37–7.31 (m, 2H), 7.29 (s, 1H), 7.13 (s, 1H), 7.09 (d, *J* = 7.7, 1.0 Hz, 2H), 7.06–7.01 (m, 1H), 5.55 (s, 2H), 4.72 (s, 2H), 3.84 (s, 3H), 3.78 (s, 3H), 2.58 (s, 6H), I.R. ν cm⁻¹ 1598 (C=N), 1572, 1516, 1479 (C=C), 1267 (N=N=C), 1337 (S–O antisym.), 1139 (S–O sym.), Analysis: C₂₁H₂₃N₅O₅S₂ (489). Calc. %: C:51.53, H:4.70, N:14.31. Found %: C:51.51, H:4.67, N:14.33.

2.1.2.14. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-cinnamyl-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVn**). Yield: 44%. M.p. 191–193 °C (CH₃OH–CH₂Cl₂); ¹H NMR (CDCl₃) δ 7.81 (d, *J* = 7.1 Hz, 2H), 7.64 (d, *J* = 16.4 Hz, 1H), 7.60 (d, *J* = 16.3 Hz, 1H), 7.52–7.40 (m, 3H), 7.30 (s, 1H), 7.16 (s, 1H), 4.73 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.62 (s, 6H), I.R. ν cm⁻¹ 1630 (CH=CH), 1600 (C=N), 1575, 1576, 1475 (C=C), 1267 (N=N=C), 1332 (S–O antisym.), 1138 (S–O sym.), Analysis: C₂₂H₂₃N₅O₄S₂ (485). Calc. %: C:54.43, H:4.74, N:14.43. Found %: C:54.45, H:4.71, N:14.39.

2.1.2.15. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-[2-(2-methoxy-phenyl)ethyl]-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVo**). Yield: 37%. M.p. 154–155 °C (CH₃OH–CH₂Cl₂), ¹H NMR (CDCl₃) δ 7.29 (s, 1H), 7.21 (m, 1H), 7.17 (d, *J* = 7.5 Hz, 1H), 7.10 (s, 1H), 6.95 (d, *J* = 8.2 Hz, 1H), 6.84 (t, *J* = 7.26 Hz, 1H), 4.67 (s, 2H), 3.84 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H), 3.29 (m, 2H), 3.01 (t, *J* = 7.5 Hz, 4H), 2.56 (s, 6H), I.R. ν cm⁻¹ 1601 (C=N), 1569, 1519, 1477 (C=C), 1267 (N=N=C), 1336 (S–O antisym.), 1139 (S–O sym.), Analysis: C₂₃H₂₇N₅O₅S₂ (517). Calc. %: C:53.38, H:5.22, N:13.53. Found %: C:53.41, H:5.19, N:13.56.

2.1.2.16. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-[2-(4-methoxy-phenyl)ethyl]-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVp**). Yield: 41%. M.p. 151–152 °C (CH₃OH–CH₂Cl₂), ¹H NMR (CDCl₃) δ 7.29 (s, 1H), 7.18 (d, *J* = 8.3 Hz, 2H), 7.11 (s, 1H), 6.83 (d, *J* = 8.1 Hz, 2H), 4.67 (s, 2H), 3.83 (s, 3H), 3.77 (s, 3H), 3.71 (s, 3H), 2.99 (t, *J* = 7.6 Hz, 2H), 2.55 (s, 6H), I.R. ν cm⁻¹ 1601 (C=N), 1569, 1519, 1477 (C=C), 1267 (N=N=C), 1336 (S–O antisym.), 1139 (S–O sym.), Analysis: C₂₃H₂₇N₅O₅S₂ (517). Calc. %: C:53.38, H:5.22, N:13.53. Found %: C: 53.35, H:5.24, N:13.50.

2.1.2.17. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3-phenylpropyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVq**). Yield: 14%. M.p. 128–129 °C (CH₃OH), ¹H NMR (CDCl₃) δ 7.30–7.26 (m, 3H), 7.23–7.17 (m, 3H), 7.12 (s, 1H), 4.69 (s, 2H), 3.82 (s, 3H), 3.77 (s, 3H), 3.04 (t, *J* = 7.4 Hz, 2H), 2.68 (t, *J* = 7.7 Hz, 2H), 2.59 (s, 6H), 2.03 (p, *J* = 7.6 Hz, 2H), I.R. ν cm⁻¹ 1600 (C=N), 1574, 1516, 1478 (C=C), 1267 (N=N=C), 1334 (S–O antisym.), 1139 (S–O sym.), Analysis: C₂₃H₂₇N₅O₄S₂ (501). Calc. %: C:55.09, H:5.38, N:13.97. Found %: C:55.11, H:5.41, N:14.01.

2.1.2.18. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(2-pyridinyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVr**). Yield: 32%. M.p. 198–199 °C (CH₃OH), ¹H NMR

(CDCl₃) δ 8.76, (s, 1H), 8.23–8.00 (m, 2H), 7.69 (s, 1H), 7.37–7.15 (m, 2H), 4.79 (s, 2H), 3.83 (s, 3H), 3.78 (s, 3H), 2.62 (s, 6H), I.R. ν cm⁻¹ 1599 (C=N), 1576, 1517, 1456 (C=C), 1270 (N=N=C), 1335 (S–O antisym.), 1137 (S–O sym.), Analysis: C₁₉H₂₀N₆O₄S₂ (460). Calc. %: C:49.56, H:4.34, N:18.26. Found %: C:49.60, H:4.31, N:18.30.

2.1.2.19. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(4-pyridinyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVs**). Yield: 37%. M.p. 231–232 °C (C₂H₅OH), ¹H NMR (CDCl₃) δ 8.85 (d, *J* = 5.0 Hz, 2H), 7.91 (d, *J* = 5.0 Hz, 2H), 7.30 (s, 1H), 7.21 (s, 1H), 4.80 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.63 (s, 6H), I.R. ν cm⁻¹ 1600 (C=N), 1561, 1507, 1473, 1411 (C=C), 1274 (N=N=C), 1337 (S–O antisym.), 1138 (S–O sym.), Analysis: C₁₉H₂₀N₆O₄S₂ (460). Calc. %: C:49.56, H:4.34, N:18.26. Found %: C:49.58, H:4.37, N:18.23.

2.1.2.20. 3-[2-(*N,N*-dimethylsulfamoyl)-4,5-dimethoxy-benzyl]-6-(3-bromo-5-pyridinyl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole (**IVt**). M.p. 227–228 °C (C₂H₅OH), ¹H NMR (CDCl₃) δ 9.10 (d, *J* = 2.0 Hz, 1H), 8.98 (d, *J* = 2.2 Hz, 1H), 8.58 (s, 1H), 7.30 (s, 1H), 7.22 (s, 1H), 4.79 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 2.63 (s, 6H), I.R. ν cm⁻¹ 1600 (C=N), 1573, 1516, 1481, 1440, 1412 (C=C), 1270 (N=N=C), 1338 (S–O antisym.), 1140 (S–O sym.), Analysis: C₁₉H₁₉BrN₆O₄S₂ (539). Calc. %: C:42.30, H:3.52, N:15.58. Found %: C:42.32, H:3.49, N:15.56.

2.2. Biological evaluation

2.2.1. In vitro anticancer activity

Twenty disubstituted triazolo-thiadiazole derivatives (**Scheme 1** and **Table 1**) were tested for anticancer activity against nine well established human cancer cell lines (4 ovarian, 2 breast, 1 prostate cancer, 1 epidermoid carcinoma and 1 leukemia) (**Table 2**). SKOV-3, OVCAR-3, UWB1.289, UWB1.289 + BRCA1 ovarian cancer cells, MCF7, T-47D breast adenocarcinoma cells, PC-3 prostate adenocarcinoma cells, A-431 epidermoid carcinoma cells and MOLT-4 T-lymphoblastic leukemia cells were treated for testing cytostatic (growth inhibition: IG50, TGI) and cytotoxic/cytocidal (IC50) activity generated by the newly synthesized compounds at concentrations of 1–100 μM. The cell lines were obtained from the American Type Culture Collection (ATCC) and were grown in different culture media according to the instructions. The MTT ((3-(4,5-imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a well-established and standard method for evaluating the cytostatic and cytotoxic activity of drugs and chemicals (**Camoutsis et al., 2005; Trafalis et al., 2005, 2004, 2006**). Briefly, the cells were plated in 96-well plate at a density of 3 × 10⁴ cells/ml per well and maintained for 72 h at 37 °C in a 5% CO₂ incubator and grown as monolayers or suspensions. After 24 h, cells were treated with 1–100 μmol/l of the compounds for 48 h. The viability of cultured cells was estimated MTT (Sigma, St Louis, Missouri, USA) metabolic assay as described previously. Absorbance of the converted dye was measured at a wavelength of 540 nm on an ELISA reader (Versamax, Orleans, USA). The mean concentrations of each drug that generated 50% or total (100%) growth inhibition (GI50 and TGI, respectively) as well as the drug concentrations that

Table 2 Description of histotypes and characteristics of the 8 human cell lines treated with the tested for antineoplastic activity newly synthesized triazolo-thiadiazole derivatives.

Cancer type	Human cell line designation	Oncogenes	Special characteristics
Ovarian adenocarcinoma	SKOV-3		Tumor Necrosis Factor; Diphtheria Toxin; Cis-platinum and Adriamycin resistant
Epithelial ovarian adenocarcinoma	OVCAR-3		Androgen/Estrogen/Progesterone receptor positive; Adriamycin, Melphalan and Cisplatin resistant
Ovarian carcinoma	UWB1.289	p53 + BRCA1 – (mutated)	Estrogen/Progesterone receptor negative/BRCA1 mutated
Ovarian carcinoma	UWB1.289 + BRCA1	p53 + BRCA1 +	Estrogen/Progesterone receptor negative
Epithelial breast adenocarcinoma	MCF7	WNT7B +	Estrogen receptor positive
Epithelial breast adenocarcinoma	T-47D	WNT7B +	Insulin-like growth factor binding proteins (IGFBP) BP-2; BP-4; BP-5 Calcitonin; androgen receptor, positive; progesterone receptor, positive; glucocorticoid; prolactin; estrogen receptor, positive
Prostate Adenocarcinoma	PC-3		Hormone resistant
Acute T-lymphoblastic leukemia	MOLT-4		Terminal deoxynucleotidyl transferase (TdT) expressed
Epidermoid carcinoma	A-431	P53 –	High expression of the Epidermal growth factor receptor (EGFR)

produced cytotoxicity against 50% of the cultured cells [(half maximal cytotoxic concentration (IC₅₀)] were calculated using the linear regression method. Using seven absorbance measurements [time 24 h (Ct₂₄), control growth 72 h (Ct₇₂), and test growth in the presence of drug at five concentration levels (Tt_{72x})], the percentage of growth was calculated at each level of the drug concentrations. The percentage growth inhibition was calculated according to National Cancer Institute (NCI) as follows: $[(Tt_{72x}) - (Ct_{24}) / (Ct_{72}) - (Ct_{24})] \times 100$ for concentrations for which $Tt_{72x} > Ct_{24}$ and $[(Tt_{72x}) - (Ct_{24}) / Ct_{24}] \times 100$ for concentrations for which $Tt_{72x} < Ct_{24}$; GI₅₀ was calculated from $[(Tt_{72x}) - (Ct_{24}) / (Ct_{72}) - (Ct_{24})] \times 100 = 50$, TGI from $[(Tt_{72x}) - (Ct_{24}) / (Ct_{72}) - (Ct_{24})] \times 100 = 0$, and IC₅₀ from $[(Tt_{72x}) - (Ct_{24}) / Ct_{24}] \times 100 = 50$. All the experiments were carried out in triplicate.

2.2.2. *In vivo acute toxicity*

For intraperitoneal (i.p.) treatment, stock solutions of the 20 tested compounds (Scheme 1 and Table 1) were prepared immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in 10% dimethylsulfoxide (DMSO). This concentration by itself produced no observable toxic effect.

C57Bl/6 female mice were used for toxicity studies. Mice were obtained from experimental section of the Hellenic Pasteur Institute.

Briefly, the acute toxicity induced by the tested compounds was determined, as previously had very well described (Trafalis et al., 2005, 2004, 2006) following a single i.p. injection into groups of ten (10) C57Bl/6 mice at four different dosages; the mice were observed for 30 days and the therapeutic dose of the compounds, which is usually defined as LD₁₀ (lethal dose for 10% of animals) as well as LD₅₀ (lethal dose for 50% of animals) was determined after graphical estimation (30-day

curves). The toxicity of the tested compounds was assessed from lethality in C57Bl/6 mice. The LD₅₀ and LD₁₀ values were estimated graphically, where the percentage of deaths due to the toxicity of each dose was shown in the ordinate, while the administered doses were indicated on the abscissa.

2.3. *In silico studies*

PharmMapper Server was used to obtain information regarding possible mechanisms behind the activity of studied compounds (Chen et al., 2011; Liu et al., 2010). PharmMapper Server is a freely accessed web-server designed to identify potential target candidates for the given probe small molecules (drugs, natural products, or other newly discovered compounds with binding targets unidentified). PharmMapper utilizes an integrated pharmacophore matching platform with statistical method for potential target identification (<http://59.78.96.61/pharmmapper/index.php>). Compounds are scored according to their fitness on the pharmacophore models. Moreover, the program encompasses z' score which is a score generated from the molecule's fit score and a library score matrix calculated beforehand. It combines the fit score and its corresponding vector in the score matrix together and normalizes it to a vector with a mean of zero and a standard deviation of one. Compared to the fit score z' -score not only applies the pharmacophore matching method but also considers statistic factors lying behind. Generally, large positive z' -score indicates high significance of the target to a query compound, as well large negative z' -score indicates that the target may not be significant enough.

LigandScout 4.0 Advanced (Wolber and Langer, 2005) was utilized to create the shared pharmacophore features of the active compounds **IVn** and **IVb** as well as those of the inactive ones, **IVe** and **IVa**. LigandScout 4.0 Advanced is available

from InteLigand, GmbH, Vienna, Austria <http://www.inteligand.com/ligandscout>.

3. Results and discussion

3.1. *In vitro* cytostatic and cytotoxic activity

The 20 novel triazolo-thiadiazole derivatives (Scheme 1 and Table 1) were tested *in vitro* against a panel of 9 well-established human cancer cell lines (Table 2). Results regarding the most active derivatives are presented in Table 3a. As it is shown, compounds **IVn** and **IVb** exhibited a very potent cytostatic and cytotoxic effect against all tested cell lines. The derivatives **IVi** and **IVk** were active but exhibited lower anticancer potency, while the compounds **IVr** and **IVa** were less active. The derivatives **IVe** and **IVc** were relatively inactive at the concentrations tested *in vitro*. Although the triazolo-thiadiazole derivatives **IVn** and **IVb** produced important cytotoxic activity, in general it was demonstrated that the tested derivatives rather hold a potential cytostatic than cytotoxic anticancer activity, probably acting like antimetabolites or targeted molecular pathway agents (Tonkinson et al., 1997; Svendsrud et al., 1997). The rest 12 triazolo-thiadiazole derivatives (**IVa**, **IVd**, **IVf**, **IVg**, **IVh**, **IVj**, **IVl**, **IVm**, **IVo**, **IVp**, **IVq**, and **IVs**) were inactive at the concentrations tested with IG50, TGI and IC50 > 100 μM in all 9 human cancer lines.

For comparison reasons, three well-established anticancer agents currently used in cancer chemotherapy were tested for cytostatic and cytotoxic effects *in vitro*, against 6 human cancer cell lines (2 ovarian, 1 prostate, 2 breast cancer and 1 leukemia)

(Table 3b). Carboplatin is a commonly used less toxic than cisplatin newer agent that exerts its antineoplastic effects acting like a non-classical alkylator interacting with DNA. Vinorelbine is a very active antitumor agent, the first 5'NOR semi-synthetic vinca alkaloid, pharmacologically acting due to inhibition of mitosis through interaction with tubulin. Pemetrexed is an evolved newer agent chemically similar to folic acid, in the class of chemotherapy drugs called folate antimetabolites, producing its effects by inhibiting three enzymes used in purine and pyrimidine synthesis: thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase. Thus, by inhibiting the formation of precursor purine and pyrimidine nucleotides, pemetrexed prevents the formation of DNA and RNA which are required for the growth and survival of cancer cells.

It is notable that all the compounds produced relatively very low acute toxicity on C57Bl/6 mice (Table 4). All LD10s from the i.p. administration of the tested triazolo-thiadiazole derivatives were over 350 mg/kg whereas LD50 s were not reached in any case. For the derivatives **IVa**, **IVc-h**, **IVj-m**, and **IVo-s**, acute toxicity was not demonstrated at the higher of the i.p. administrated dosage and LD10 s and LD50 s were not reached (> 500 mg/kg).

Thus, the new triazolo-thiadiazole derivatives that were tested and specifically **IVn**, **IVb**, **IVi**, **IVk**, **IVr** and **IVa** generated potent cytostatic and cytotoxic effects against all tested human cancer cell lines *in vitro* and better or equal activity in comparison with three well-established antitumor drugs in current cancer chemotherapy. This is important activity comes together with the generation of relatively very low acute

Table 3a Growth inhibition/cytostatic (GI50 and TGI) and cytotoxic/cytotoxic (IC50) anticancer effects induced by the 8 newly synthesized triazolo-thiadiazole derivatives on nine human cancer cell lines.

	Compound	GI50 (μM)	TGI (μM)	IC50 (μM)		GI50 (μM)	TGI (μM)	IC50 (μM)		GI50 (μM)	TGI (μM)	IC50 (μM)
UWB1.289 + BRCA1	IVn	12	28	42	OVCAR-3	5	12	25	SKOV-3	8	16	32
	IVb	8	64	>100		3	32	>100		8	38	>100
	IVi	8	64	>100		8	68	>100		12	56	>100
	IVk	16	>100	>100		5	86	>100		6	>100	>100
	IVr	>100	>100	>100		24	>100	>100		20	>100	>100
	IVa	76	>100	>100		65	>100	>100		80	>100	>100
	IVe	90	>100	>100		97	>100	>100		>100	>100	>100
	IVc	85	>100	>100		95	>100	>100		>100	>100	>100
UWB1.289	IVn	12	56	>100	MCF-7	15	45	>100	T-47D	8	37	>100
	IVb	30	50	>100		22	50	>100		27	55	>100
	IVi	13	>100	>100		18	>100	>100		24	>100	>100
	IVk	30	>100	>100		40	>100	>100		28	>100	>100
	IVr	>100	>100	>100		>100	>100	>100		>100	>100	>100
	IVa	56	>100	>100		65	>100	>100		52	>100	>100
	IVe	>100	>100	>100		>100	>100	>100		>100	>100	>100
	IVc	>100	>100	>100		>100	>100	>100		>100	>100	>100
PC-3	IVn	12	31	85	MOLT-4	4	21	36	A-431	11	28	78
	IVb	20	45	>100		6	45	78		16	36	>100
	IVi	27	>100	>100		7	55	>100		19	75	>100
	IVk	44	>100	>100		10	>100	>100		35	>100	>100
	IVr	>100	>100	>100		85	>100	>100		>100	>100	>100
	IVa	62	>100	>100		42	>100	>100		74	>100	>100
	IVe	>100	>100	>100		55	>100	>100		>100	>100	>100
	IVc	>100	>100	>100		94	>100	>100		>100	>100	>100

Table 3b Growth inhibition/cytostatic (GI50 and TGI) and cytotoxic/cytotoxic (IC50) anticancer effects induced by 3 well-established cancer therapeutics anticancer agents.

Human cancer cell lines	Anticancer agents								
	Carboplatin			Pemetrexed			Vinorelbine		
	IG50 (μM)	TGI (μM)	IC50 (μM)	IG50 (μM)	TGI (μM)	IC50 (μM)	IG50 (μM)	TGI (μM)	IC50 (μM)
OVCAR-3	66	> 100	> 100	> 100	> 100	> 100	< 1	< 1	70
SCOV-3	> 100	> 100	> 100	> 100	> 100	> 100	< 1	3	79
PC-3	> 100	> 100	> 100	> 100	> 100	> 100	< 1	28	87
MCF-7	> 100	> 100	> 100	< 1	> 100	> 100	< 1	21	65
T-47D	> 100	> 100	> 100	> 100	> 100	> 100	< 1	52	> 100
MOLT-4	74	> 100	> 100	< 1	> 100	> 100	< 1	5	> 100

Table 4 Acute toxicity of the compounds in C57Bl/6. The acute toxicity induced by the tested compounds following a single intraperitoneal (i.p.) injection into groups of ten (10) C57Bl/6 mice at four different dosages was determined; LD50 and LD10 = lethal doses for 50% and 10% of the population of the treated mice. Where LD50s and LD10s were not reached it is indicated as over 500 mg/kg (> 500), the higher dosage that was administered.

Compounds	LD50 (mg/kg)	LD10 (mg/kg)
IVn	375	> 500
IVb	430	> 500
IVi	480	> 500
IVk	> 500	> 500
IVr	> 500	> 500
IVa	> 500	> 500
IVe	> 500	> 500
IVc	> 500	> 500

toxicity on C57Bl/6 mice *in vivo*, lending a significant clinical practice accrued therapeutic ratio.

These results provide evidence that the newly synthesized compounds, especially **IVn** and **IVb** are of high interest for cancer therapeutics, since they provide very low acute toxicity and conclusively low systemic toxicity in correlation with high anticancer activity *in vitro* thus relatively high therapeutic ratios.

3.2. Proposed mechanisms behind activity

Triazolo-thiadiazole derivatives exhibit antitumor activity by targeting and inhibiting various molecular pathways and crucial macromolecules in cancer cells. Molecular targets and pathways involved in the biological activities of these compounds are protein kinases (tyrosine, serine and threonine kinases), phosphatidylinositol-3 lipid kinases, metalloproteinases, cells defective in the von Hippel-Lindau gene, Bcl-2 family proteins, histone acetyltransferases and deacetylases and cancer cells with high expression in Cancer 1 (Hec1) (Leoni et al., 2014a, 2014b; Morigi et al., 2015).

Implementation of PharmMapper on two active (**IVb** and **IVn**) versus two inactive (**IVa** and **IVe**) compounds has resulted in several significant and insignificant protein targets which are presented in Table 5. These target proteins may partially

explain the mechanism behind the activity of **IVn** and secondly **IVb** on ovarian and epithelial ovarian carcinoma and adenocarcinoma cell lines (Table 3). As can be observed, apoptotic protease-activating factor 1 (APAF1) has been matched as the most significant target for **IVn** (z' score equal to 3.81), and it is estimated to be significant for **IVb** (z' score equal to 2.40) but rather insignificant for the activity of **IVa** (z' score equal to 0.80) and **IVe** (z' score equal to 1.26). Indeed, the regulation of APAF-1 activity is suggested to be important for apoptosis in some ovarian cancers (Wolf et al., 2001). Furthermore, in ovarian carcinoma, the APAF1 gene has been found to be active (Wolf et al., 2001). APAF-1 is the structural core of the apoptosome. Oligomeric APAF-1 mediates the cytochrome c-dependent autocatalytic activation of pro-caspase-9 (Apaf-3), leading to the activation of caspase-3 and apoptosis.

A second common significant protein target for **IVn** (z' score equal to 3.13) and **IVb** (z' score equal to 2.73) and insignificant for **IVa** (z' score equal to 0.47) and **IVe** (z' score equal to -0.05) is the Tyrosine-protein kinase HCK. Tyrosine kinase plays an essential role for the selection and maturation of developing T-cell and in mature T-cell function. It is constitutively associated with the cytoplasmic portions of the CD4 and CD8 surface receptors. Tyrosine-protein kinase has been correlated with leukemia.

The cell division protein kinase 2 (CDK2), another possible target for **IVb** (z' score equal to 2.89), is hyperactivated and most often associated with amplification and/or overexpression of its partner cyclins A and E, particularly in breast cancer, ovarian and endometrial carcinomas, lung and thyroid carcinoma, melanoma and osteosarcoma (Peyressatre et al., 2015).

Compound **IVb** displays also significant z' score (2.46) for the matrix metalloproteinase 3 (MMP3) which along with MMP2 and MMP9 is representative protease known to be involved in ovarian metastasis (Wu et al., 2014).

Heat shock protein HSP 90 is a possible target especially for **IVn** (z' score equal to 2.75). The suppression of HSP90 signals the inhibition of multi-receptor tyrosine kinases (RTKs) and results in profound pro-apoptotic and anti-proliferative effects in individual ovarian cancer cell lines and primary tumors (Jiao et al., 2011).

Finally, lymphocyte-specific protein tyrosine kinase Lck, which belongs to the Proto-oncogene tyrosine-protein kinase (Src) family, has been found significant target for **IVn** (z' score equal to 2.37 versus 0.46, 0.53 and 0.51 for **IVb**, **IVa** and **IVe** respectively), and may explain its activity on Acute

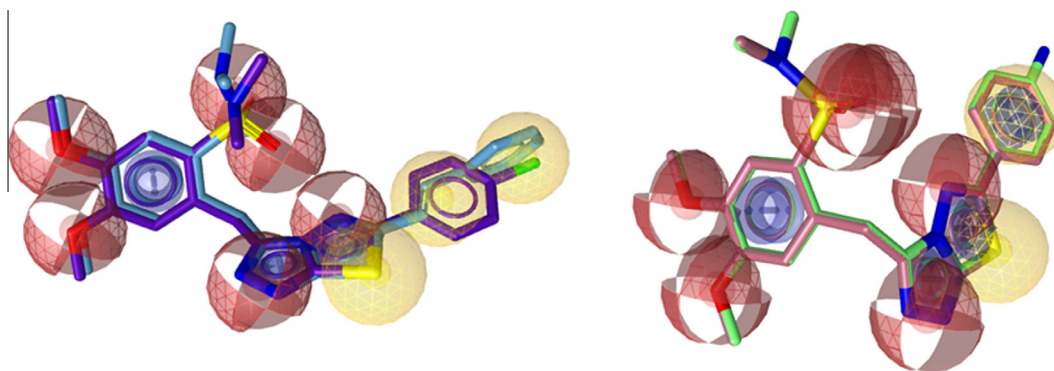


Figure 1 (left) Shared pharmacophore features of the overlaid active molecules **IVn** (light blue) and **IVb** (purple) include six hydrogen bond acceptors (red spheres), three aromatic rings (blue rings) and three hydrophobic regions (yellow spheres); (right) shared pharmacophore features of the overlaid inactive molecules **IVe** (light green) and **IVa** (pink) including six hydrogen bond acceptors (red spheres), four aromatic rings (blue rings) and two hydrophobic regions (yellow spheres). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5 Results from PharmMapper matching platform indicating potential protein target candidates.

Target protein	Compound			
	z' scores			
	IVn	IVb	IVa	IVe
Cell division protein kinase 2 P24941 PDB:1DI8	0.02	2.89	2.58	2.56
Stromelysin-1 MMP3 HUMAN PDB: 1CIZ	0.49	2.46	0.11	0.53
Apoptotic protease-activating factor 1 APAF HUMAN PDB: 1Z6T	3.81	2.40	0.80	1.26
Tyrosine-protein kinase HCK HCK HUMAN PDB: 1QCF	3.13	2.73	0.47	-0.05
Heat shock protein HSP 90-alpha HS90A HUMAN PDB: 1UYG	2.75	1.12	2.02	2.30
Proto-oncogene tyrosine-protein kinase LCK LCK HUMAN PDB: 1QPC	2.37	0.46	0.53	0.51

High significance values appear in bold.

T-lymphoblastic leukemia cell lines (Table 3). Lck phosphorylates specific tyrosine residues in other proteins involved in the intracellular signaling pathways of lymphocytes. Upregulation of Lck is observed in lymphoma, breast and colon cancer (Patil and Kundu, 2005).

In an effort to further explore structure-activity relationship features, two active (**IVb** and **IVn**) and two inactive (**IVa** and **IVe**) analogues have been selected and subjected to ligand based pharmacophore model generation (Fig. 1). Results indicate that the shared pharmacophore model of the active compounds is characterized by one more hydrophobic feature which might contribute positively to their binding at a hydrophobic core of the biological target.

4. Conclusions

The role of the thiadiazole and triazole heterocycles as a versatile scaffold for the synthesis of new derivatives has been presented. Some of the newly synthesized 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole derivatives and more specifically **IVn** and **IVb** showed substantial cytostatic and cytotoxic antineoplastic activity *in vitro* and they produced relatively low acute toxicities *in vivo*. Apoptotic protease-activating factor 1 (APAF1), tyrosine-protein kinase HCK, cell division protein kinase 2 (CDK2) and matrix metalloproteinase 3 (MMP3) may be involved in the biological activities of active analogues on ovarian and epithelial ovarian carcinoma and adenocarcinoma cell lines. Moreover, the lymphocyte-specific protein tyrosine kinase Lck may explain the activity of **IVn** on Acute T-lymphoblastic leukemia cell lines.

Conclusively, many of these derivatives might be useful to increase the effects of standard drugs and improve the therapeutic response in cancer patients.

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