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RESEARCH ARTICLE

Novel hybrids of drug with bioactive heterocycles for enhancing biological activity

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Abstract A novel series of aceclofenac hybridised with 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles were designed using molecular hybridization approach and synthesised **6a–j**. The structural integrity was confirmed by analytical methods. The hybrid molecules were subjected to in vitro cytotoxic studies against four human cancer cell lines PA-1, OAW-42, T47-D and MCF-7 by MTT assay method. The results indicate that the hybrid molecules bearing halogen on phenyl ring in 6th position of triazolo-thiadiazole exhibited significant cytotoxic activity. The test compounds were also screened for antifungal activity against two strains.

Keywords Triazolo-thiadiazole · Cytotoxic · Molecular hybrids

Introduction

In the last few decades, triazoles have received much significant attention in the field of medicinal chemistry because of their diversified biological properties (Balaban et al. 2004; Khan et al. 1978; Goswami et al. 1984; Allen et al. 1973; Hart 1999). The triazole nucleus containing drugs are available in market as antifungal agents such as fluconazole, itraconazole, ravuconazole and posaconazole. Triazole derivatives such as 3-aryl amino-5-(hetero) aryl-1,2,4-triazole acts as polymerization inhibitor by binding to the colchicines binding site on tubulin (Block and Beale 2004) and 3-s-alkylated-5-(hetero) aryl-1,2,4-triazole is Somatostatin sst_2/sst_5 binding agonist (Lesy et al. 2007).

1,3,4-Thiadiazole entity acts as an important component in number of drugs and exhibit wide spectrum of biological activities (Haydar et al. 2006; Dmitry and Douglas 2007; Siddiq and Alam 2009, Schenone et al. 2006; Hill 1980; Nelson et al. 1977; Tsukamoto et al. 1975, Priyabrata et al. 2008). The biological activities of [1,3,4]thiadiazoles may be due to the presence of the toxophoric =N–C–S moiety (Richard et al. 1956). Recently bis-triazolo thiadiazoles showed anti-cancer activity (Holla et al. 2002; Chowrasia et al. 2013). Moreover the available literature, in vitro and in vivo data suggests that certain antioxidant, selectively hinder the growth of tumour cells and change the intra cellular redox state, thereby enhancing the effects of cytotoxic therapy (Sunil et al. 2010; Lamson and Brignall 1999; Conklin 2000; Conklin 2002). In this regard, heteroaromatic hybrid structures like triazolothiadiazoles are extensively used as scaffolds because of the diverse biological activities including potential antitumor agent (Matao 1957), CNS depressant (Omar and Abolwaya 1986), antibacterial, antifungal agent (Invidiata et al. 1996), antiviral agent (El-Khawass et al. 1989), anticancer agent (Roman et al. 2007; Imtiaz et al. 2014).

In addition, these heterocyclic structures are widely used as pharmacophore for the discovery of potent and specific biologically active agents. Combretastatin, a naturaly occurring small bioactive structure possesses potent anti-mitotic activity. The *cis*-stilbenes form is more cytotoxic than the more stable trans-forms (Odlo et al. 2008; Liu et al.

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2009). The literature reveals that the replacement of the double bond bridge with a rigid triazole ring exhibited potent anticancer activity. Recently (Pei et al. 2012) introduced 3-thio-1,2,4-triazole ring to the lock cis-type bridge and resulted with high cytotoxic activity. Furthermore, literature survey revealed that modification of aryl propionic acid derivatives of Non Steroidal Anti-Inflammatory Drugs (NSAIDs) results in various biological activities such as anti-inflammatory, antibacterial and antiviral. The anti-tumor effects of NSAIDs have been attributed mainly to the inhibition of cyclooxygenase (COX1/2) and their anti-inflammatory effects, albeit COX independent inhibition of tumor cell proliferation and induction of apoptosis have been also reported (Hanif et al. 1996; Zhou et al. 2010).

The molecular hybridisation approach involves the synthesis of strategically designed newer breeds of structures having different characteristic features. The drug design considers the following key points: (a) the desire substructures linked by a spacer; (b) both substuructures fused without spacer; (c) the desired activities are merged in a new structure (Kakwani et al. 2011; Viegas-Junior et al. 2007).

Non-steroidal anti-inflammatory drugs such as diclofenac exhibit potent anticancer effects (Gottfried et al. 2013). Modification of the carboxyl function of aryl acid derivatives of non- steroidal anti inflammatory drugs (NSAIDs) resulted in bioactive moieties. Prompted by these findings the present work was planned with the modification of NSAIDs aceclofenac. The incorporation of triazolo-thiadiazole moiety in aryl propionic acid group of diclofenac enhances anticancer activity (Ilango and Valentina 2010). Based on the our earlier observations and in continuation of our research work on synthesis of small bio active heterocycles and development of novel hybridized molecules (Ilango et al. 2009; Ilango and Valentina 2010; Ilango et al. 2015) in the present investigation we envisaged to hybridise three pharmacophore i.e., 1,2,4-triazolo, 1,3,4-thiadiazole and aceclofenac (Fig. 1). For the first time to the best of our knowledge we herein report the synthesis and cytotoxic evaluation of acelofenac hybridised with 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles. The study aimed to address the following points (1) To synthesise the active pharmacophore hybridised with aceclofenac, (2) To understand the contribution of electron donor, acceptor, H bond donor, H bond acceptor and aromatic ring substituted in thiadiazole ring. (3) To evaluate for cytotoxic and antifungal activity.

Results and discussion

Chemistry

The starting material 2-[(2′,6′- dichloro phenyl) amino] phenyl acetoxy acetic acid **1** upon reflux with ethanol gave ethyl 2-[(2′,6′-dichloro phenyl) amino] phenyl acetoxy acetate **2** (Scheme 1). The ethyl ester **2** were converted to hydrazide **3** by refluxing with hydrazine hydrate which on further reaction with carbon disulphide in alcoholic potassium hydroxide yields potassium dithiocarbazinate **4**. The required 2-[(2′,6′- dichloro phenyl) amino] phenyl acetoxy methyl]-4-amino-5-mercapto-(4H)-1,2,4-triazole **5** were obtained by refluxing compound **4** with aqueous hydrazine hydrate. The triazole was further converted to the target compounds 3[2-(2′,6′-dichloro phenyl) amino phenyl acetoxy methyl]- 6 (substituted aryl)-1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles **6a–j** by condensation with various aromatic acid in presence of phosphorous oxychloride ($POCl_3$). The $POCl_3$ activates the carbonyl group of aromatic acid and increases its electrophilicity to enhance the formation of thiadiazole. The excess of phosphorus oxychloride was neutralized with potassium hydroxide and potassium carbonate by raising the pH to 8. The IR spectra of compound **5** showed characteristic absorption bands, one which appearing at 2608 cm^{-1} was attributed to SH and the other at 3403 cm^{-1} was assigned to NH_2. Similarly, the ^{1}HNMR spectra of the compound **5** showed two characteristic signals at δ 13.9 ppm and the other δ 5.75 ppm, due to SH and NH_2 protons respectively confirms the formation of triazole. The absence of these absorption (SH and NH_2) in the compounds **6a–j** established that the triazoles had converted to triazolo-thiadiazole by reacting with the –COOH group of various acids. The structures of newly synthesized compounds **6a–j** were characterized by IR, ^{1}HNMR, ^{13}CNMR, MS and elemental analysis. The presence of ester group in the target compounds were recognized by the two characteristic IR frequencies one owing to C=O between 1796 and 1730 cm^{-1} and other to C–O group between 1173 and 1050 cm^{-1}. In the ^{1}HNMR spectra of the compounds **6a–j**, the signal appeared between δ 5.1 and 5.5 ppm integrating for two protons was assigned to the $–COOCH_2–$ groups on the 3rd position of triazolo-thiadiazole ring. The peaks belonging to the same group was observed in ^{13}CNMR spectrum at δ 50.7–51.0 ppm for methylene carbon and at δ 174.0–177.1 ppm for carbonyl carbon of ester. The results obtained in elemental analysis were within 0.4 % of the theoretical value. The molecular ion peaks obtained were in good agreement with the molecular weight of the compounds. All the synthesized compounds **6a–j** exhibited satisfactory spectral data consistent with their structure.

In vitro cytotoxic activity

The synthesized triazolo-thiadiazole derivatives **6a–j** were evaluated for in vitro cytotoxic activity and IC_{50} values were calculated by MTT assay method against human cancer cell lines, PA-1, OAW-42, T47-D and MCF-7. On

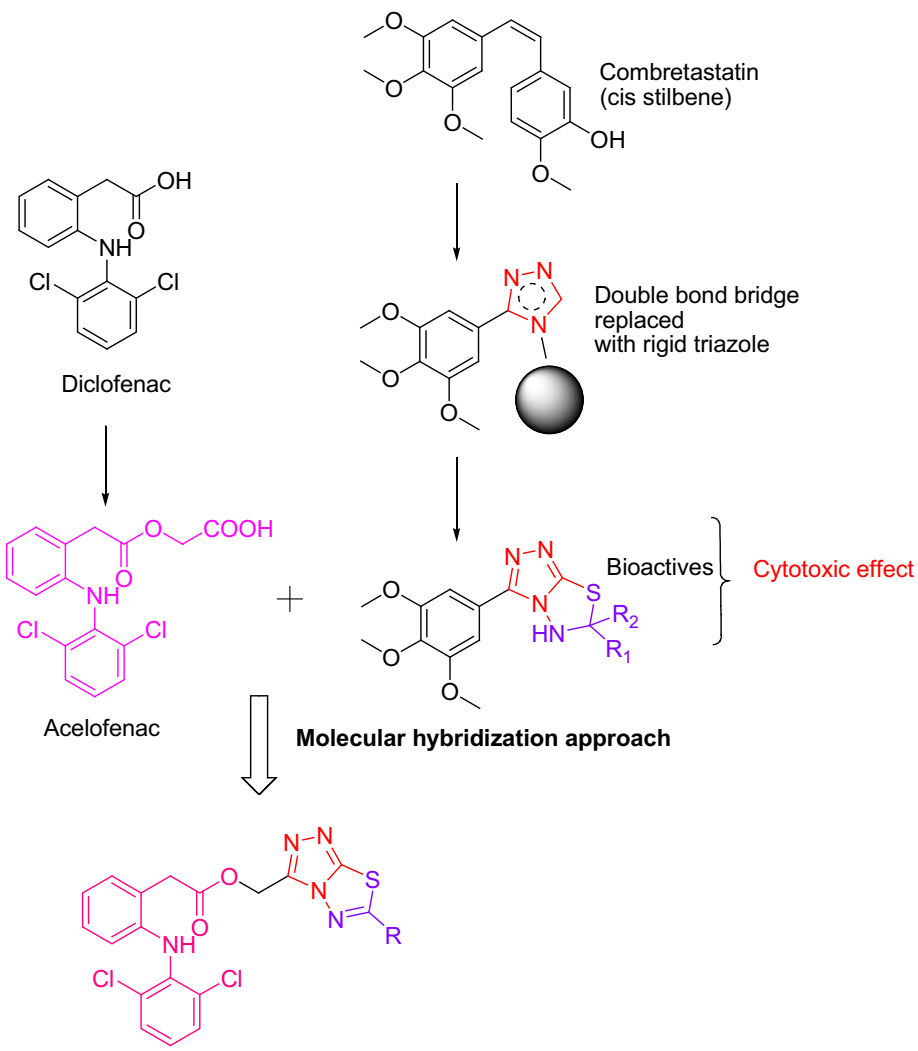


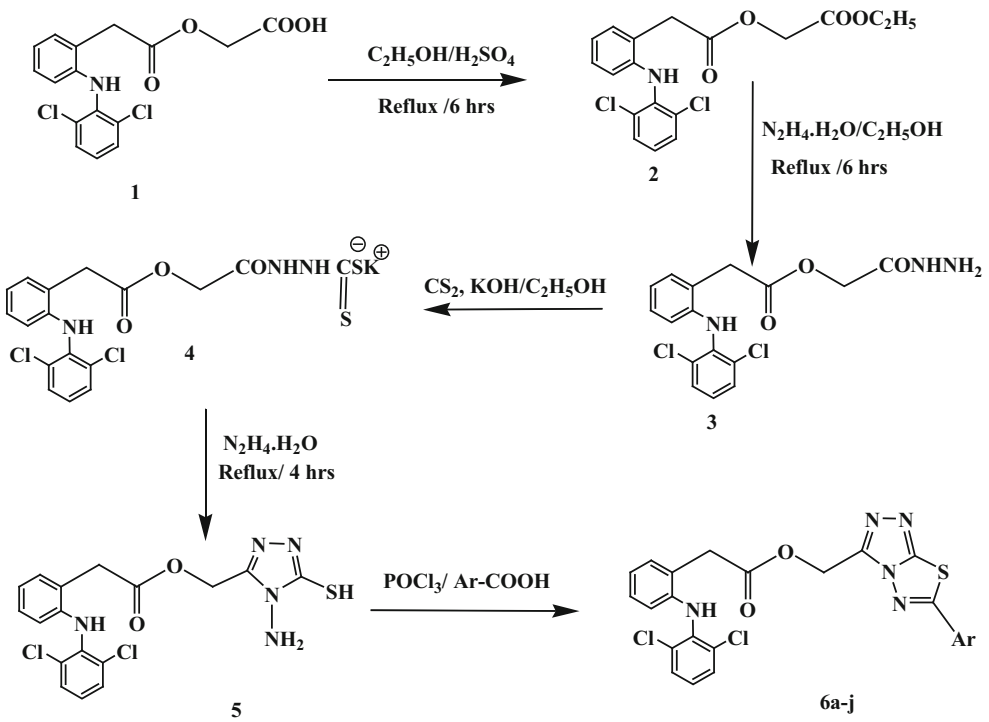
Fig. 1 Design strategy of target compounds **6a–j**

comparing with the standard drug, the test compounds **6e**, **6a** and **6g** displayed higher cytotoxicity against PA-1 ovarian cancer cell line.

The presence of chlorine and bromine on the aromatic ring in test compounds **6g**, **6a** and **6e** showed good activity against MCF-7 cell lines. Test compound **6g** exhibited IC_{50} value 0.67 µM against breast cancer cell line MCF-7. The chloro phenyl substituted compound **6a** exhibited better activity among the series against T47-D cell lines. However none of the compounds showed significant activity against OAW-42 cell lines. From the SAR study of the test compounds against all these cell lines, it was found that the presence of halo group in the phenyl ring at the 6th position exhibited better cytotoxic activity. The presence electronegative atoms such as chloro, bromo is required for better activity against PA-I. The presence of hydroxyl and hydrophobic methyl group showed good cytotoxic effect against PA-I. The substitution in the ring like $–NO_2$, –OH, $–OCH_3$, $–SO_3H$ and disubstitution resulted in moderate to poor activity. The replacement of the aromatic hydrocarbon with heterocyclic pyridine showed moderate activity against PA-I, T47-D and MCF-7. The position of halogen atom does not make much difference.

Antifungal activity

The titled compounds **6a–j** was also screened for antifungal activity against two fungal strains viz *C. albicans* and *A. niger* by broth dilution method. The compounds **6f** (MIC value 6.25 µg/ml) exhibited twofold better activities and **6h** (MIC value12.5 µg/ml) displayed equipotent activity against *C. albicans* with reference to standard drug Ketaconazole. The compounds **6d**, **6f** and **6h** showed MIC values in the range of 3.12–6.25 µg/ml against *A. niger*. The SAR study of the compounds **6a–j** showed that the compounds endowed with nitro and methoxy phenyl ring in



Scheme 1 Synthetic route for target compounds

6th position of triazolo-[3,4-b]-1,3,4-thiadiazole showed MIC value >100. The presence of hydroxy phenyl group in 6th position of the lead moiety enhances the activity.

Materials and methods

Melting point was determined on Veego digital melting point apparatus and was uncorrected. IR spectra were recorded using potassium bromide on a Perkin Elmer FTIR Spectrometer.^{1}H-NMR spectra were recorded on Bruker Spectrometer (400 MHz) in $CDCl_3$ using TMS as an internal standard and ^{13}C-NMR spectra were recorded on Bruker Spectrometer (100 MHz) in $CDCl_3$ using TMS as an internal standard. Mass spectra were recorded on LC-MSD Trap-SL 2010A-Shimadzu. Micro analysis was performed on a Perkin Elmer-240 CHN elemental analyzer.

Chemistry

*Procedure for the synthesis of 2-(2-[(2′,6′-Dichloro phenyl) amino] phenyl acetoxyacetate (**2**)*

Aromatic acid **1** (0.01 mol) were taken in 50 ml of ethanol and 1 ml of sulphuric acid was added and refluxed for 6 h. The progress of the reaction was monitored by TLC. Upon completion, the excess of solvent was removed under reduced pressure, cooled and filtered to yield compound **2**.

*2-(2-[(2′,6′-Dichloro phenyl) amino] phenyl acetoxyacetate (**2**)*

White solid colour; yield: 65 %; m.p: 165–166 °C; R_f: 0.71; FT-IR (KBr) νmax: 3323, 2950, 1746, 1627, 1268, 1076, 744 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 7.4–7.9 (m, 7H, ArH), 5.4 (s, 2H, $–CH_2CO$), 4.2 (s, 1H, NH), 4.0 (q, 2H, $–OCH_2CH_3$), 3.4(s, 2H, $–OCH_2–$), 1.25 (t, 3H, $–OCH_2CH_3$).

*Procedure for the synthesis of 2-[(2′,6′-Dichloro phenyl) amino] phenyl acetoxy acetyl hydrazide (**3**)*

A mixture of compound **2** (0.01 mol) and hydrazine hydrate (0.02 mol) in 50 ml ethanol was refluxed for 6 h. The reaction mixture was left overnight and solid obtained **3** was collected by flask evaporator and recrystallized.

*2-[(2′,6′-Dichloro phenyl) amino] phenyl acetoxy acetyl hydrazide (**3**)*

Yellow solid crystals; yield: 75 %; m.p: 190–196 °C; R_f: 0.82; FT-IR (KBr) νmax: 3375, 3146, 2719, 1717, 1617, 1602, 1491, 1249, 1060, 798 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 7.8 (s, 2H, NH_2), 6.6–7.4 (m, 7H, ArH), 5.1 (s, 2H, $–CH_2CO$), 4.35 and 3.85 (s, 2H, 2(NH)), 3.85 (s, 2H, $–OCH_2–$).

*Procedure for the synthesis of 3-[2-[(2′,6′-Dichloro phenyl) amino] phenyl acetoxy methyl]-4-amino-5-mercapto-(4H)-1,2,4-triazole (**5**)*

A solution of 50 ml of alcoholic potassium hydroxide (0.03 mol) was cooled in an ice bath followed by addition of compound **3** (0.016 mol) and carbon disulphide (0.025 mol) and stirred for 12 h at room temperature. The precipitated potassium thiocarbamate **4** was filtered, washed with ethanol, dried and used for the next step. To potassium thiocarbamate **4** (0.02 mol) was added distilled water (8 ml) followed by hydrazine hydrate (0.02 mol) and refluxed for 4 h. The homogeneous reaction mixture turned to green with evolution of hydrogen sulphide gas. The reaction mixture was then cooled to room temperature and diluted with water. Upon on acidification with acetic acid, the triazole **5** precipitated out. The TLC solvent system were *n*-hexane: ethyl acetate: formic acid (5:4:1).

*3-[2-[(2′6′-Dichloro phenyl) amino] phenyl acetoxy methyl]-4-amino-5-mercapto(4H)-1,2,4-triazole (**5**)*

White solid; yield: 66 %. m.p.: 158–159 °C; R_f: 0.64; FT-IR (KBr) νmax: 3403, 3146, 2863, 2608, 1742, 1604, 1220, 1094, 772 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 13.9 (s, 1H, SH), 7.2–8 (m, 7H, ArH), 5.75 (s, 2H, NH_2), 5.1 (s, 2H, $–CH_2CO$), 4.2 (s, 1H,NH), 3.45 (s, 2H, $–OCH_2–$).

*General method for the synthesis of 3,6-disubstituted-1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles (**6a–j**)*

An equimolar concentration of compound **5** and appropriate aromatic acids in phosphorus oxychloride 10 ml was refluxed for 6 h. The completion of reaction was monitored by TLC using precoated silica gel G plate with chloroform: methanol: acetic acid (6:3:1) as solvent system. Then the reaction mixture was cooled to room temperature and poured onto crushed ice with stirring. The mixture was neutralized with potassium carbonate and potassium hydroxide solutions till pH 8 to remove the excess of phosphorus oxychloride. The solid obtained was collected by vacuum distillation, dried and recrystallized from methanol.

*3-[2-(2′,6′-Dichloro phenyl) amino phenyl acetoxy methyl]-6-(2-chloro phenyl)-1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole (**6a**)*

Yellowish white solid; yield: 70 %; m.p: 184–185 °C; R_f: 0.64; FT FT-IR (KBr) νmax: 3465, 3032, 2892, 1731, 1513, 1360, 1238, 1090, 782, 747, 628 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.5–8.1 (m, 11H, ArH), 5.2 (s, 2H, $–COOCH_2–$); 4.15 (s, 1H, NH), 3.45 (s, 2H, $–CH_2$-COO–); ^{13}CNMR δ ($CDCl_3$, ppm):147.3 (C-1), 124.5 (C-2), 131.4 (C-3), 124.7 (C-4), 131.0 (C-5), 123.8 (C-6), 152.9 (C-7), 119.6 (C-8), 126.6 (C-9), 140.8 (C-10), 128.5 (C-11), 119.0 (C-12), 128.6 (C-13), 134.5 (C-14), 129.2 (C-15), 134.9 (C-16), 121.4 (C-17), 131.8 (C-18), 161.4 (C-19), 172.0 (C-20), 173.8 (C-21), 35.9 (C-22), 174.3 (C-23), 51.4 (C-24); m/z: 546(M^{+2}, 50 %); Anal. Calcd. for $C_{24}H_{16}Cl_3N_5O_2S$ (544.84): C, 54.28; H, 2.90; N, 14.39. Found: C, 54.31; H, 2.89; N, 14.41.

*3-[2-(2′,6′-Dichloro phenyl) amino phenyl acetoxy methyl]-6-(2-nitrophenyl)-1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole (**6b**)*

Brownish orange crystals; yield: 64 %; m.p:164–165 °C; R_f: 0.70; FT-IR (KBr) νmax: 3400, 3116, 2977, 1731, 1526, 1463, 1483, 1346, 1312, 1263, 1173, 788, 717, 671 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 7.0–8.5 (m, 11H, aromatic), 5.3 (s, 2H,$–COOCH_2–$), 4.25 (s, 1H, NH), 3.4 (s, 2H, $–CH_2COO–$);^{13}CNMR δ ($CDCl_3$, ppm): 147.4 (C-1), 124.2 (C-2), 131.7 (C-3), 125.0 (C-4), 131.6 (C-5), 123.9 (C-6), 151.9 (C-7), 120.3 (C-8), 127.1 (C-9), 141.5 (C-10), 130.1 (C-11), 121.0 (C-12), 122.8 (C-13), 1446.8 (C-14), 130.7 (C-15), 135.6 (C-16), 120.7 (C-17), 132.2 (C-18), 161.2 (C-19), 171.8 (C-20), 173.4 (C-21), 35.7 (C-22), 174.0 (C-23), 50.9 (C-24); m/z: 555.6 (M^{+1},42 %); Anal. Calcd. for $C_{24}H_{16}Cl_2N_6O_4S$ (554.01): C, 53.16 %; H, 2.84 %; N, 16.90 %. Found: C, 53.24 %; H, 2.90 %; N, 16.81 %.

*3-[2-(2′,6′-Dichloro phenyl)amino phenyl acetoxy methyl]-6-(3-methoxy phenyl)-1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole (**6c**)*

Yellow solid;yield: 69 %; m.p:174–146 °C; R_f: 0.58; FT-IR (KBr) νmax: 3418, 3075, 2861, 1730, 1462, 1291, 1360, 1320,1093, 1038, 788, 740, 670 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.7–8.1 (m, 11H, Ar), 5.3 (s, 2H,$–COOCH_2–$), 4.5 (s, 1H, NH), 3.45 (s, 2H, $CH_2COO–$), 3.2 (s, 3H, OCH_3); ^{13}CNMR δ ($CDCl_3$, ppm): 143.2 (C-1), 124.3 (C-2), 130.0 (C-3), 124.8 (C-4), 128.9 (C-5), 122.0 (C-6), 153.2 (C-7), 119.5 (C-8),121.3 (C-9), 143.9 (C-10), 120.1 (C-11), 147.9 (C-12), 121.5 (C-13),153.7 (C-14), 119.8 (C-15),160.8 (C-16),127.3 (C-17),134.5 (C-18), 170.6 (C-19), 172.8 (C-20), 173.2 (C-21), 33.2 (C-22), 175.8 (C-23), 50.7 (C-24), 57.3 (C-25); m/z: 540.8 (M^{+1}, 36 %); Anal. Calcd. for $C_{25}H_{19}Cl_2N_6O_3S$ (539): C, 57.27: H, 3.55; N, 14.52. Found: C, 57.34; H, 3.52; N, 14.59.

*3-[2-(2′,6′-Dichloro phenyl) amino phenyl acetoxy methyl]-6-[2-(3-hydroxy benzene sulphonic acid)]-1,2,4 triazolo-[3,4-b]-1,3,4-thiadiazole (**6d**)*

Orange solid; yield: 69 %; m.p:115–116 °C; R_f: 0.63; FT-IR (KBr) νmax: 3545, 3215, 2980, 1751, 1533, 1351, 1301, 1239, 1170, 1091, 1018, 782, 748, 670 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.3–7.9 (m, 10H, aromatic), 6.3 (s, 1H, –OH), 5.2 (s, 2H, $–COOCH_2–$), 4.45 (s, 1H, NH), 3.7 (s, 2H, $–CH_2COO–$), 2.1 (s, 1H, $-SO_3H$); ^{13}CNMR δ ($CDCl_3$, ppm):148.3 (C-1), 124.9 (C-2), 130.5 (C-3), 124.0 (C-4), 130.7 (C-5), 123.0 (C-6), 153.2 (C-7), 119.1 (C-8), 127.8 (C-9), 140.0 (C-10), 129.8 (C-11), 120.0 (C-12), 115.8 (C-13),1534.2 (C-14), 130.9 (C-15), 135.9 (C-16), 138.0 (C-17), 132.8 (C-18), 161.5 (C-19), 170.3 (C-20), 172.8 (C-21), 35.3 (C-22), 174.4 (C-23), 52.1 (C-24); m/z: 607 (M^{+2},75 %); Anal. Calcd. for $C_{24}H_{17}Cl_2N_5O_6S_2$ (605):C, 48.18; H, 2.76; N, 12.77. Found: C, 48.23; H, 2.82; N, 12.80.

*3-[2-(2′,6′-Dichloro phenyl)amino phenyl acetoxy methyl]-6-(3-bromo phenyl)-1,2,4 triazolo-[3,4-b]-1,3,4-thiadiazole (**6e**)*

Pale yellow powder; yield: 61 %; m.p:169–170 °C; R_f: 0.80; FT-IR (KBr) νmax: 3435, 3232, 2916, 1732, 1511, 1360, 1237, 1032, 782, 744, 670, 540 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.5–8.0 (m, 11H, ArH), 5.5 (s, 2H, $–COOCH_2–$), 4.3 (s, 1H, NH), 3.45 (s, 2H, $–CH_2COO–$); ^{13}C-NMR δ ($CDCl_3$, ppm):147.5 (C-1), 123.9 (C-2), 131.2 (C-3), 124.2 (C-4), 131.6 (C-5), 123.5 (C-6), 153.4 (C-7), 119.8 (C-8), 126.7 (C-9), 140.6 (C-10), 130.2 (C-11), 121.0 (C-12), 129.2 (C-13), 132.2 (C-14), 122.9 (C-15), 125.9 (C-16), 130.2 (C-17), 127.2 (C-18), 161.6 (C-19), 171.5 (C-20), 172.2 (C-21), 36.2 (C-22), 175.2 (C-23), 52.3 (C-24); m/z: 605(M^{+2},67 %); Anal. Calcd. for $C_{24}H_{16}BrCl_2N_5O_2S$ (603): C, 49.74; H, 2.66; N, 13.18. Found: C, 49.80; H, 2.71; N, 13.15.

*3-[2-(2′,6′-Dichloro phenyl)amino phenyl acetoxy methyl]-6-(3-hydroxy phenyl)- 1,2,4 triazolo-[3,4-b]-1,3,4-thiadiazole (**6f**)*

White crystals;yield: 57 %; m.p:138–139 °C; R_f: 0.73; FT-IR (KBr) νmax: 3572, 3078, 2977, 1737, 1539, 1306, 1209, 1172, 1107, 1052, 783, 747, 671 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.7–8.1 (m, 11H,ArH), 6.1 (s, 1H, –OH), 5.4 (s, 2H, $–COOCH_2–$), 4.8 (s, 1H, NH), 3.45 (s, 2H, $–CH_2COO–$); ^{13}C-NMR δ ($CDCl_3$, ppm):148.6 (C-1), 125.4 (C-2), 130.9 (C-3), 123.8 (C-4), 129.0 (C-5), 123.0 (C-6), 152.5 (C-7), 119.7 (C-8), 127.8 (C-9), 140.7 (C-10), 129.8 (C-11), 121.2 (C-12), 130.6 (C-13), 115.8 (C-14), 152.5 (C-15), 114.5 (C-16), 130.3 (C-17), 127.2 (C-18), 163.1 (C-19), 172.0 (C-20), 174.1 (C-21), 35.7 (C-22), 175.2 (C-23), 51.8 (C-24); m/z: 527 (M^{+2}, 49 %); Anal. Calcd. for $C_{24}H_{17}Cl_2N_5O_3S$ (525) C, 54.42; H, 3.23; N, 14.95 Found: C, 54.39; H, 3.29; N, 14.90.

*3-[2-(2′,6′-Dichloro phenyl)amino phenyl acetoxy methyl]-6-(2,4-dichloro phenyl)-1,2,4 triazolo-[3,4-b]-1,3,4-thiadiazole (**6g**)*

Pale yellow crystals; yield: 60 %; m.p: 206–207 °C; R_f: 0.51; FT-IR (KBr) νmax: 3411, 3084, 2861, 1732, 1240, 1543, 1361, 1343, 1173, 791, 748, 671 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.4–8.5 (m, 10H,ArH), 5.5 (s, 2H, $–COOCH_2–$), 4.55 (s, 1H, NH), 3.65 (s, 2H, $–CH_2COO–$); ^{13}CNMR δ ($CDCl_3$, ppm):148.3 (C-1), 124.9 (C-2), 130.5 (C-3), 124.0 (C-4), 130.7 (C-5), 123.0 (C-6), 153.2 (C-7), 119.1 (C-8), 127.8 (C-9), 140.0 (C-10), 129.8 (C-11), 120.0 (C-12), 128.9 (C-13), 134.8 (C-14), 130.9 (C-15), 135.9 (C-16), 120.1 (C-17), 132.8 (C-18), 162.8 (C-19), 171.0 (C-20), 173.2 (C-21), 36.7 (C-22), 175.8 (C-23), 51.0 (C-24); m/z: 580 (M^{+1}, 45 %); Anal. Calcd. for $C_{24}H_{15}Cl_4N_5O_2S$ (579): C, 50.69; H, 2.51; N, 13.44.Found: C, 50.74; H, 2.59; N, 13.40.

*3-[2-(2′, 6′-Dichloro phenyl) amino phenyl acetoxy methyl]-6-(2, 4-dihydroxy phenyl)-1, 2, 4 - triazolo-[3,4-b]-1,3,4-thiadiazole (**6h**)*

Pale yellow powder;yield: 68 %; m.p: 150–152 °C; R_f: 0.57; FT-IR (KBr) νmax: 3497, 3103, 2931, 1710, 1265, 1484, 1463,1323, 1150, 1031, 790, 749, 671 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.4–8.1 (m, 10H,ArH), 5.3 and 5.4 (s, 2H, 2(OH)), 5.1 (s, 2H, $–COOCH_2–$), 4.35 (s, 1H, NH), 3.7 (s, 2H, $–CH_2COO–$); ^{13}C-NMR δ ($CDCl_3$, ppm):146.3 (C-1), 123.8 (C-2), 130.7 (C-3), 123.1 (C-4), 131.3 (C-5), 124.6 (C-6), 151.2 (C-7), 118.9 (C-8), 126.1 (C-9), 141.4 (C-10), 130.0 (C-11), 120.6 (C-12), 158.5 (C-13), 103.4 (C-14), 103.9 (C-15), 155.3 (C-16), 108.0 (C-17), 113.2 (C-18), 161.5 (C-19), 171.2 (C-20), 174.1 (C-21), 35.9 (C-22), 174.1 (C-23), 51.8 (C-24); m/z: 542 (M^{+1},52 %); Anal. Calcd. for $C_{24}H_{17}Cl_2N_5O_4S$ (541): C, 54.55; H, 3.12; N, 14.46. Found: C, 54.63; H, 3.19; N, 14.50.

*3-[2-(2′,6′-Dichloro phenyl)amino phenyl acetoxy methyl]-6-(2,4-dimethoxy phenyl)-1,2,4 triazolo-[3,4-b]-1,3,4-thiadiazole (**6i**)*

White powder; yield: 71 %; m.p: 121–122 °C; R_f: 0.5; FT-IR (KBr) νmax: 3434, 3094, 2961, 1730, 1548, 1391, 1360, 1240,1053, 1019, 789, 749, 670 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.9–8.0 (m, 10H, ArH), 5.1 (s, 2H, $–COOCH_2–$), 4.3 (s, 1H, NH), 3.5 (s, 2H, $–CH_2COO–$), 3.2

and 3.3 (s, 6H, 2 (–OCH_3)); ^{13}C-NMR δ ($CDCl_3$, ppm):149.1 (C-1), 123.5 (C-2), 131.2 (C-3), 122.4 (C-4), 132.2 (C-5), 124.7 (C-6), 153.2 (C-7), 118.4 (C-8), 126.8 (C-9), 142.8 (C-10), 132.8 (C-11), 122.0 (C-12), 126.1 (C-13), 164.6 (C-14), 102.9 (C-15), 165.3 (C-16), 125.7 (C-17), 132.5 (C-18), 163.4 (C-19), 170.9 (C-20), 172.2 (C-21), 35.5 (C-22), 177.1 (C-23), 53.6 (C-24); m/z: 571 (M^{+2}, 26 %); Anal. Calcd.for $C_{26}H_{21}Cl_2N_5O_4S$ (569): C, 56.26; H, 3.74; N, 13.67. Found: C, 56.34; H, 3.79; N, 13.59.

3-[2-(2′,6′-Dichloro phenyl)amino phenyl acetoxy methyl]-6-(pyridin-3-yl)-1,2,4- triazolo-[3,4-b]-1,3,4-thiadiazole ***(6j)***

Yellow crystals; yield: 65 %; m.p: 235–236 °C; R_f: 0.68; FT-IR (KBr) νmax: 3484, 3151, 3089, 1731, 1486, 1360, 1301, 1291, 1039, 782, 748, 669 cm^{-1}; 1H NMR ($CDCl_3$, 300 MHz): δ = 6.4–8.6 (m, 10H, ArH), 5.35 (s, 2H, –$COOCH_2$–), 4.5 (s, 1H, NH), 3.7 (s, 2H, –CH_2COO–);^{13}-CNMR δ ($CDCl_3$, ppm):147.5 (C-1), 125.1 (C-2), 131.5 (C-3), 123.4 (C-4), 131.3 (C-5), 121.6 (C-6), 154.5 (C-7), 120.7 (C-8), 127.8 (C-9), 144.0 (C-10), 130.8 (C-11), 120.0 (C-12), 124.9 (C-13), 150.3 (C-14), 150.8 (C-15), 122.6 (C-16), 134.9 (C-17), 162.8 (C-18), 171.4 (C-19), 173.2 (C-20), 36.8 (C-21), 174.1 (C-22), 52.6 (C-23); m/z: 514 (M^{+2}, 61 %); Anal. Calcd. for $C_{23}H_{15}Cl_2N_6O_2S$ (512): C, 55.64; H, 3.11; N, 18.54. Found: C, 55.72; H, 3.19; N, 18.50.

In vitro cytotoxic activity

Tumor cell lines used in this study are two breast human cell lines T47-D (ductal carcinoma) and MCF-7 (adreno carcinoma) and two ovarian cancer cell lines PA-1 (terato carcinoma) and OAW-42 (ovarian cystadeno carcinoma). All the cells were obtained from cell line bank of National Center of Cellular Sciences (NCCS), Pune, India. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at pH 7.4, supplemented with 10 % heat-inactivated fetal bovine serum, Ciprofloxacin 5 μM and Gentamycin 40 μM in a humidified incubator at 37 °C and 5 % CO_2 atmosphere. The cells from a particular cell line when in log phase of growth are trypsinized, counted in a haemocytometer and adjusted to 10^4 densities in a DMEM medium per plates and then inoculated in 96-well plates. The cells are treated in 0.01–100 μM concentrations of test compounds for specified duration (1–4 days). After which the MTT dye was added in each well and plates were incubated at 37 °C for 4 h in a CO_2 incubator. The plates are then taken out of incubator and dark blue colored formazan crystals are thoroughly dissolved in 800μL dimethyl sulphoxide (DMSO) at room temperature for 15 min. The plates are then read on an ELISA reader (Textan) at 550 nm. Doxorubicin 10 μM was used as a standard drug for comparison. The 50 % of the cell viability (IC_{50}) was calculated with a dose dependent curve and given in the Table 1.

Antifungal activity

Fungal species used for the study is *Candida albicans* (ATCC 11231) and *Aspergillus niger* (*NCIM 627*). Normal saline was used to make a suspension of spores of fungal strain for lawning. A loopful of particular fungal strain was transferred to 3 ml medium to get a suspension of corresponding species. The two-fold dilution of synthesized compound solutions was prepared (400, 200,100…3.12 μg/

Table 1 In vitro cytotoxic activity of 1,2,4 triazolo-[3,4-b]-1, 3,4-thiadiazole **6a–j**

Compound	Ar	IC_{50} in μM[a]			
		PA-1	OAW-42	T47-D	MCF-7
6a	2-Chloro phenyl	**0.68** ± 0.63	75 ± 0.14	12 ± 0.45	0.75 ± 0.19
6b	2-Nitro phenyl	3.20 ± 0.52	>100	92 ± 0.22	1.60 ± 0.45
6c	3-Methoxy phenyl	1.80 **±** 0.87	74 ± 0.25	80 ± 0.26	3.20 ± 0.95
6d	3-Hydroxy benzene sulphonic acid	0.81 ± 0.56	63 ± 0.66	46 ± 0.51	8.20 ± 0.35
6e	3-Bromo phenyl	**0.64 ±** 0.44[b]	25 ± 0.55	36 ± 0.38	0.83 ± 0.74
6f	4-Hydroxy phenyl	0.84 ± 0.94	81 ± 0.43	40 ± 0.72	5.40 ± 0.96
6g	2,4-Dichloro phenyl	**0.72** ± 0.88[b]	73 ± 0.21	50 ± 0.52	**0.67** ± 0.24[b]
6h	3,4-Dihydroxy phenyl	2.51 ± 0.57	96 ± 0.26	55 ± 0.13	5.70 ± 0.69
6i	3,4-Dimethoxy phenyl	3.60 ± 0.62	89 ± 0.18	82 ± 0.10	5.30 ± 0.11
6j	3-Pyridyl	1.50 ± 0.37	60 ± 0.64	16 ± 0.33	7.50 ± 0.38
Doxourubin		0.74	0.62	0.77	0.72

[a] Data expressed as mean ± SEM (n = 3)

[b] Entries in bold font indicates better activity than reference drug doxourubin

Table 2 Antifungal activity of 1,2,4 triazolo-[3,4-b]-1,3,4-thiadiazole **6a–j**

Compound code	Antifungal activity MIC in μg/ml	
	C. albicans	*A. niger*
6a	25	12.5
6b	100	200
6c	>200	100
6d	25	**3.12**[b]
6e	50	25
6f	**6.25**[b]	**6.25**[b]
6g	25	12.5
6h	12.5	**6.25**[b]
6i	25	50
6j	100	25
Ketoconazole	12.5	6.25

[a] Data expressed as mean ± SEM (n = 3)

[b] Entries in bold font indicates equivalent and better activity than reference drug ketoconazole

ml). The standardized suspensions of the test organisms were inoculated into a series of tubes containing two fold dilutions and incubated at 37 °C for 48 h. The MIC of the compound was recorded as the lowest concentration in the tubes with no turbidity (i.e., no growth) of inoculated fungi and given in the Table 2.

Conclusion

In conclusion, we report for the first time synthesis of acelofenac hybridized with 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazole as cytotoxic agent with antifungal activity. All the compounds displayed appreciable cytotoxic activity against both ovarian and breast cancer cell lines. Three test compounds exhibited excellent cytotoxic activity against PA-1 ovarian cancer cell lines and one compound against MCF-7 breast cancer cell lines. Structure-biological relationship of title compounds against PA-I and MCF-7 cell lines showed that, halogen substituted on phenyl ring at 6th position of 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles is important for activity when compared with the standard drug. Compounds such as **6e**, **6a** and **6g** showed better activity against PA-I cell lines than the standard drug. However one of the compound **6g** showed better activity than the standard in MCF-7 cell lines. The compound with hydroxyl phenyl ring in the 6th position of 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles displayed two-fold better antifungal activity on comparison with the standard drug against both fungal strains.

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