



CHEMISTRY & BIOLOGY INTERFACE

An official Journal of ISCB, Journal homepage; www.cbijournal.com

Triazolo-thiadiazepine as a potent apoptotic DNA degradation agent and MetAP2 inhibitor against human breast adenocarcinoma cells

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Received 21 January 2014; Accepted 16 February 2015

Abstract: Breast cancer is the second leading cause of cancer deaths in females. In the present research work, two new chalcones, (2*E*)-1-(4-fluorophenyl)-3-[3-substituted]-1*H*-pyrazol-4-yl]prop-2-en-1-ones have been synthesized by Claisen-Schmidt reaction between pyrazole carbaldehydes and 4-fluoroacetophenone. A novel series of 3-[(substituted)methyl]-6-(4-fluorophenyl)-8-(3-substituted-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4] thiadiazepines were prepared by the base catalysed reaction between chalcones and 5-substituted-4-amino-3-mercapto-1,2,4-triazoles and were characterized by elemental analysis and spectroscopic techniques. 3-[(2-methylphenoxy)methyl]-6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4e**), displayed excellent cytotoxic activity in MCF-7 cells with very low IC₅₀ of 5 µg/mL compared to the standard drug Doxorubicin, in the MTT assay. **4e** at 10 µg/mL exhibited a highly significant concentration dependent increase in tail length and olive tail moment leading to apoptotic DNA degradation in comet assay. MetAP2, a bifunctional enzyme involved in angiogenesis, found overexpressed in cancer cells and essential for the progression of solid tumors was used as the target for molecular docking studies to investigate the inhibitory action of **4e**. The favourable binding interactions as **4e** positions itself into the active site of MetAP2 and its compliance with Ro5 suggests it to be a potent chemotherapeutic agent in the treatment of breast adenocarcinoma. Chalcones revealed better antioxidant properties in *in-vitro* DPPH and ABTS scavenging assays than thiadiazepines.

Keywords: Thiadiazepine, cytotoxicity, apoptosis, MetAP2, antioxidant.

Introduction

Breast cancer is the most common cancer in

women worldwide. Chemotherapy either used alone or along with other modalities is central to its treatment. The development of selective and

effective antineoplastic drugs with minimum toxicity is of utmost importance in synthetic medicinal chemistry [1]. Synthetic chemistry has been employed to generate drug leads for anticancer therapy through specific identification of new cancer-related targets such as proteins or nucleic acids. MetAP2, a member of the M24 family of intracellular metalloenzymes, controls the removal of methionine from the amino-terminus of nascent cellular proteins and promotes further amino terminal modifications [2]. It mediates protein synthesis and thereby regulates cell growth and proliferation [3, 4]. MetAP2 is up-regulated during cell proliferation [5] and is found elevated in several forms of cancers [6]. Different Src family kinases (SFKs), ADP ribosylation (ARFs) factors and eukaryotic transcription elongation factor-2 which are involved in cancer metastasis are substrates of MetAP2. Recent studies indicate that antisense of MetAP2 induces apoptosis in hepato-carcinoma cells in rat [7]. Lowering of MetAP2 leads to inhibition of protein synthesis and cell growth due to high levels of eIF2 α phosphorylation and thereby initiates apoptosis [8]. MetAP2 inhibition by anti-angiogenic agent TNP470 has been shown to activate p53 for cell cycle arrest by alkylating a histidine residue in the enzyme active center, thereby preventing cell proliferation [9]. Anti-angiogenic natural products such as Fumagillin, Belorinib and ovalicin inhibit MetAP2. As MetAP2 plays a critical role in cancer, it is a potential target for therapeutic mediation in cancer chemotherapy [10].

Chalcones is an important class of natural products belonging to flavonoid family which has interesting cytotoxic, anti-inflammatory, antidiabetic and antioxidant properties [11-13]. Certain chalcones with hydroxyl substituents are reported to exhibit enhanced cytotoxic activity towards tumor cells. They act as biochemical intermediates in the polyketide biosynthetic pathway. Thiadiazepines, a seven membered heterocyclic moiety, constitute an

important and interesting diverse biological group with significant antibacterial, antifungal, analgesic, anticoagulant, and antidepressant properties [14-19]. They are excellent charge generating agents [20] and are also testified to be intermediates for preparations of substituted caprolactams used for treating HIV disease [21].

1,2,4-triazole is a unique moiety which displays a broad spectrum of biological activities [22-26]. There are several marketed pharmaceutical preparations containing the triazole ring like Terconazole, Itraconazole, Trazodone, Triazolam. Anastrozole and Letrozole, are third generation aromatase inhibitors used in the treatment of metastatic breast cancer with triazole as the active metabolite [27]. 1,2-pyrazole ring is another moiety that exhibits a wide range of medicinal properties and is among the topmost in the list of best-selling pharmaceutical formulations [28-31].

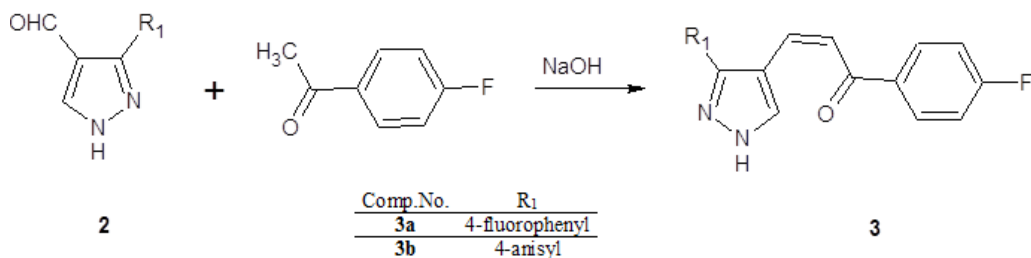
Several studies have revealed that antioxidants augment the effect of chemotherapy in cancer, as they inhibit oxidative damage induced by reactive oxygen species [32, 33]. Most of these chemotherapeutic agents are toxic at their optimum doses and are expensive. Hence, there is an urgent need to develop newer drugs with higher efficiency. Keeping these objectives in mind, and in continuation of our work on biologically active novel classes of heterocycles [34-36], we hereby report the synthesis, characterization, antioxidant and anticancer activities, *in vitro* and *in silico* of some novel pyrazole containing chalcones and triazolo-thiadiazepines incorporating three pharmacologically active heterocyclic systems.

Materials and methods

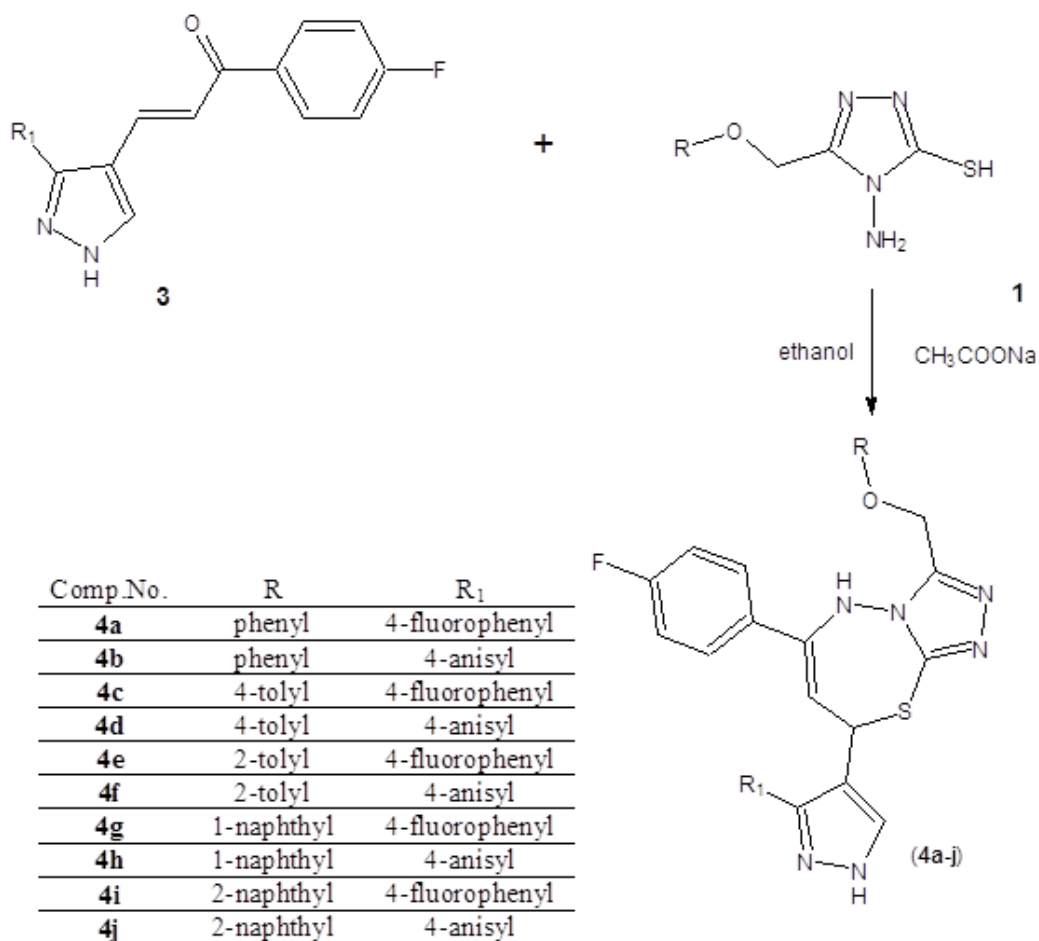
Chemistry: 3-substituted-4-amino-5-mercapto-1,2,4-triazoles **1** were prepared by multistep reactions from substituted phenols based on literature [37]. Vilsmeier Haack reaction of substituted semicarbazones was carried out

using phosphorous oxychloride and dimethyl formamide. The reaction mixture on hydrolysis gave 3-substituted-1*H*-pyrazole carbaldehydes **2** [38]. Claisen-Schmidt condensation of 3-substituted-1*H*-pyrazole carbaldehydes **2** with 4-fluoro acetophenone yielded two new (2*E*)-1-(4-fluorophenyl)-3-[3-substituted)-1*H*-pyrazol-4-yl]prop-2-en-1-ones (chalcones)

3a-b [33] (Scheme-1). 3-[(substituted)methyl]-6-(4-fluorophenyl)-8-(3-substituted-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazepines **4a-j** were synthesized by the reaction between 3-substituted-4-amino-5-mercapto-1,2,4-triazoles **1** and (2*E*)-1-(4-fluorophenyl)-3-[3-substituted)-1*H*-pyrazol-4-yl]prop-2-en-1-ones **3a-b** [20] (Scheme-2).



Scheme 1: Synthetic route for the preparation of chalcones



Scheme-2: Synthetic route for the preparation of thiadiazepines

Thin layer chromatography was performed to follow the reaction progress as well as to confirm the purity of the compounds. The elemental analysis of the newly synthesized compounds was carried out in Flash thermo 1112 series CHN analyser. The IR spectra in KBr pellets and ^1H -NMR spectra with TMS as internal standard and DMSO-d_6 as solvent were recorded in a Shimadzu FTIR 8400S spectrophotometer and 400 MHz AV500 NMR spectrometer respectively. The mass spectra were taken in a Shimadzu GCMS-QP5050 mass spectrometer. Melting points of chalcones and thiadiazepines were determined by open capillary method and were uncorrected.

Anticancer activity: *In vitro* anticancer studies were carried out in MCF-7 cells purchased from NCCS, Pune and cultured at 37 °C in Dulbecco's Modified Eagles Medium (DMEM) with 10 % Fetal Bovine Serum (FBS) in 5 % CO_2 atmosphere.

MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay): Chalcones and randomly selected six thiadiazepines were screened for their anticancer activities in MCF-7 cell lines. The stock solution was prepared by dissolving the compounds in 10 % dimethyl sulphoxide (DMSO) and diluting with phosphate buffer saline (PBS). 1×10^4 cells/well was seeded in well plates, incubated for 24 h. The cells were treated with 0.1, 1, 10 and 100 $\mu\text{g/mL}$ of chalcones and thiadiazepines. 5×10^{-3} mol of MTT reagent was added after 24 h and incubated further for 4 h. Formazan crystals developed were dissolved in hydrochloric acid (0.4 N): isopropanol (1:24) mixture (0.1 mL) and the absorbance was measured at 570 nm in an ELISA plate reader [39]. Cells treated with doxorubicin and appropriate concentration of DMSO was used as standard and control respectively.

Comet assay (Single cell gel electrophoresis): Comet assay was performed to evaluate the

extent of DNA damage caused by different concentrations of thiadiazepines in an individual MCF-7 cell. 1×10^6 cells were treated with thiadiazepines for 24 h, washed and 200 μL of cell suspension in low melting agarose (LMA) was layered on to the slides precoated with agarose (1.5 %). The slides were allowed to undergo lysis in 2.5 % M NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1 % Troton X – 100 mixture at pH 10 for 1 h at 4 °C. Further the slides were equilibrated in alkaline buffer (30 mM NaOH, 1 mM EDTA) of pH 13 at 4 °C, electrophoresed at 0.86 V/cm, neutralized, washed and dried. Then the slides were stained with ethidium bromide (EtBr, 150 μL 1X), images taken and viewed under a fluorescent microscope (20 x, Olympus BX-51, Japan). Tail length (TL), and olive tail moment (OTM) were analyzed using image analyzer CASP software version 1.2.2 [40].

Molecular modeling and docking studies:

Docking plays an important role in rational drug design as it frequently predicts the binding affinity and orientation of the ligand towards the protein target. The crystal structure of MetAP2 from human was retrieved from the PDB database (www.pdb.org) with the corresponding entry code 2EA4 complexed with A797859 (F79) with a resolution of 2.35 Å. The protein structure was prepared by removing all water molecules and appended hydrogen atoms using the Accelrys Discovery Studio ver 1.7 (Accelrys, Inc., San Diego), licensed to Manipal Institute of Technology, Manipal University, India. The ligands were built; energy minimized using CHARMM force field, and docked into the active site using the complexed 2-(2-aminoethoxy)-3-ethyl-6-[[4-(4-fluorophenyl)sulfonyl]amino} benzoic acid (F79) as a reference molecule. One hundred conformations of ligands and their docking modes into the active pocket were generated using CDOCKER protocol. The best conformation was chosen based on most favorable conformation in terms of lowest ligand-receptor binding free energies.

Antioxidant studies

DPPH radical scavenging assay: DPPH radical scavenging mediated by hydrogen transfer is based on its decolorization in presence of antioxidants. Different concentrations of chalcones / thiadiazepines were added to respective wells in a 96-well plate, followed by 100 μ L of DPPH. The absorbance was measured at 517 nm using an ELISA plate recorder, after 20 min incubation in the dark. The control wells were treated with 100 μ L each of methanol and DPPH. Ascorbic acid was used as the standard [41]. The radical scavenging capacity of the test compounds was calculated using the following equation.

$$\% \text{ scavenging} = \left\{ \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right\} \times 100$$

ABTS radical scavenging assay: The electron transfer ability of synthesized compounds was studied using ABTS assay. In a 96-welled micro plate, 40 μ L of chalcones / thiadiazepines / ascorbic acid, 200 μ L of methanol and 30 μ L of ABTS solution were added. The plate was then incubated at 37 $^{\circ}$ C for 20 min and the optical density was measured at 690 nm [42].

Results and discussion

Chalcones showed a strong IR absorption band at 1670 cm^{-1} characteristic of C=O stretching vibration. The absorption shifted to a lower frequency due to its conjugation with the neighboring double bonds. The $^1\text{H-NMR}$ spectra of chalcones showed two equally spaced doublets between 7.6-7.8 ppm for CH=CH protons, as they were conjugated. This equal spacing indicated that the protons are on adjacent carbons or coupled. The coupling constants are found to be greater than 15 Hz which specified that the protons are in *trans* position. The molecular ion peaks are in accordance with

their respective molecular weights.

The amino and mercapto groups in substituted triazole served as efficient nucleophilic centers for the synthesis of fused triazolo-thiadiazepine rings. Nucleophilic addition of mercapto group of substituted mercapto triazole occurred at the β -carbon atom of the substituted α,β -unsaturated ketone. This was followed by condensation of the carbonyl group with the amino group of the triazole to give the seven membered thiadiazepine ring.

Formation of condensed thiadiazepines was confirmed from their IR spectra which exhibited the disappearance of bands at 1670 cm^{-1} and 2550 cm^{-1} due to C=O stretching vibration of chalcones and SH stretching vibration of 5-[(substituted)methyl]-4-amino-3-mercapto-1,2,4-triazoles respectively. The peak at δ 8.4 in the $^1\text{HNMR}$ spectra was due to N-H protons of pyrazole ring. The 5-H proton of pyrazole resonated at 7.6 ppm. The peaks at δ 6.9-8.2 were due to protons of aromatic rings. $^1\text{H-NMR}$ spectra of fused thiadiazepines showed the $>\text{CH-S}$ proton at 5 ppm and $-\text{CH=}$ proton at 7.5 ppm. The NH proton of thiadiazepine ring was observed at 8.5 ppm. The solvent peak due to DMSO- d_6 was observed at 2.49. The mass spectra showed molecular ion peaks which were in accordance with their respective molecular masses. The fragment observed at $m/z = 95$ was due to the fluorophenyl ion.

The IC_{50} for the standard drug doxorubicin was found to be 18 $\mu\text{g/mL}$ in MTT assay. **3a** with IC_{50} value of 39.55 ± 4.40 was found to show better anticancer activity between the two chalcones (IC_{50} of **3b** = 43.28 ± 4.24). Thiadiazepines displayed significant cytotoxic activity as opposed to chalcones. The results of MTT assay of thiadiazepines are given in Table-1.

Among the six thiadiazepines screened in MTT assay, **4a**, **4b** and **4h** exhibited good cytotoxicity

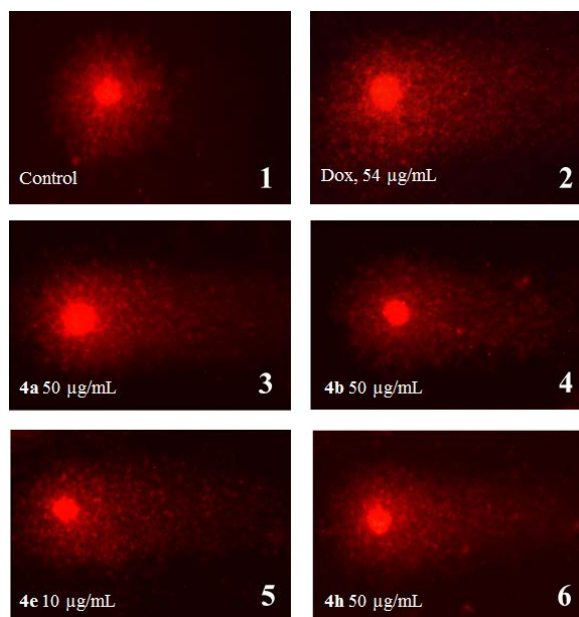
Table 1: Cytotoxic studies of thiadiazepines in MCF-7 cell lines

Comp No.	Vehicle Control (% survival)	0.1 $\mu\text{g/mL}$ (% survival)	1 $\mu\text{g/mL}$ (% survival)	10 $\mu\text{g/mL}$ (% survival)	100 $\mu\text{g/mL}$ (% survival)	IC ₅₀ $\mu\text{g/mL}$
4a	100 $\pm$ 6	87.97 $\pm$ 2.73	81.89 $\pm$ 9.98	65.68 $\pm$ 1.78	9.64 $\pm$ 3.07	20
4b	100 $\pm$ 7.53	72.10 $\pm$ 4.14	78.32 $\pm$ 8.34	69.66 $\pm$ 6.45	2.19 $\pm$ 0.68	20
4c	100 $\pm$ 7.53	79.98 $\pm$ 9.58	79.94 $\pm$ 1.03	83.13 $\pm$ 3.85	19.68 $\pm$ 2.53	33
4e	100 $\pm$ 7.53	75.96 $\pm$ 8.17	75.38 $\pm$ 1.89	39.16 $\pm$ 2.55	4.01 $\pm$ 1.54	5
4g	100 $\pm$ 7.53	82.67 $\pm$ 3.46	87.94 $\pm$ 8.83	72.56 $\pm$ 7.60	23.62 $\pm$ 2.75	30
4h	100 $\pm$ 7.53	71.07 $\pm$ 5.88	72.56 $\pm$ 7.84	67.55 $\pm$ 5.91	0.29 $\pm$ 0.47	20

with IC₅₀ of 22, 22 and 20 $\mu\text{g/mL}$ respectively. **4e** with 2-methylphenyl and 4-fluorophenyl groups as substituents exhibited excellent cytotoxic behavior with an IC₅₀ of 5 $\mu\text{g/mL}$, which was one-fourth as that of presently employed DNA intercalating antineoplastic drug doxorubicin having adverse cardiac toxicity.

The images of comet assay for control, cells treated with doxorubicin (0.1 μM , 54 $\mu\text{g/mL}$), **4a** (50 $\mu\text{g/mL}$), **4b** (50 $\mu\text{g/mL}$) **4e** (10 $\mu\text{g/mL}$) and **4h** (50 $\mu\text{g/mL}$) are depicted in Fig.1. The images of cells treated with doxorubicin, **4a**,

4b, **4e** and **4h** showed the comet appearance. The control cells did not show any signs of comet pattern. There was dose related rise in tail length and olive tail moment when treated with **4a**, **4b**, **4e** and **4h** (Fig. 2). The quantified increase in DNA damage suggested that all four thiadiazepines induced dose dependent disintegration of chromosomal DNA leading to apoptosis. **4e** presented maximum apoptotic DNA damage at a very low concentration of 10 $\mu\text{g/mL}$, in comparison with doxorubicin (54 $\mu\text{g/mL}$). This is in agreement with the results observed in MTT assay.

**Fig.1** Comet pattern observed in MCF-7 cells due to DNA degradation

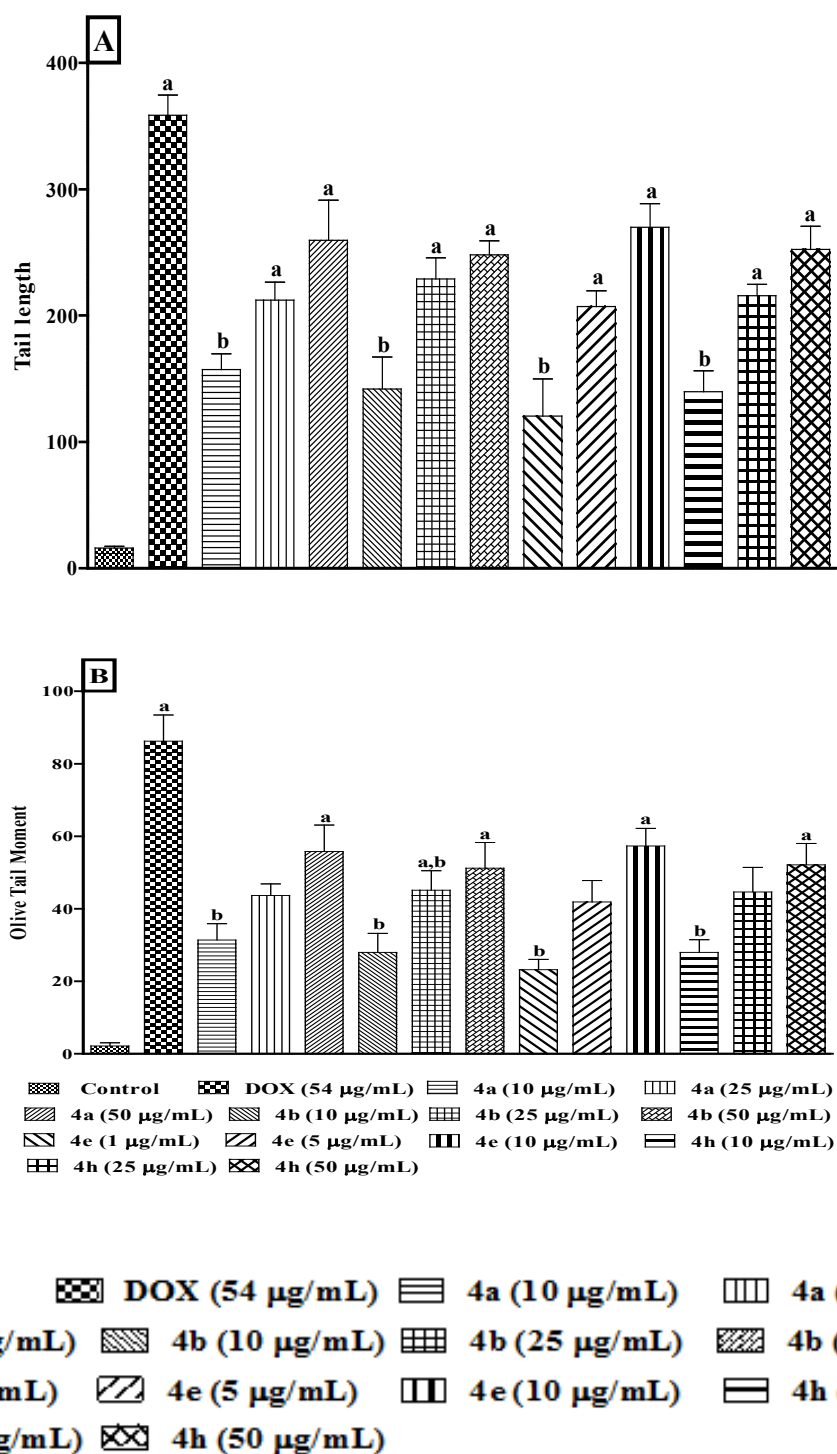


Fig.2 Concentration dependent tail length (A) and olive tail moment (B) in MCF-7 cells

The high anticancer activity of **4e** could be attributed to the presence of fluorine atoms and the methyl group in the *ortho* position of phenyl ring. The increased dipole moment of C-F bond might have enhanced the intermolecular interactions through hydrogen bonds as visualized in the molecular docking studies. This might have led to the antineoplastic property of the molecule. The presence of methyl groups might have augmented the lipophilicity, and also it could have rendered the molecule more compact or globular, thereby increasing membrane permeability as observed in **4e**-MetAP2 docking studies. Thus the presence of fluorine atom and methyl group could have radically modified the bioavailability of the compound and thus its efficacy as a potent chemotherapeutic agent against human breast adenocarcinoma.

The docked conformers of the reference molecule, F79 and thiadiazepine **4e** are presented in Fig.3A. The binding energy for the most stable pose between **4e** - MetAP2 was found to be -51.5336 Kcal/mol, indicating a stable conformation of **4e** with MetAP2. The alignment of **4e** in the active site of MetAP2 with the bonding interactions with the surrounding

amino acids in the enzyme pocket is depicted in Fig.3B. Short range hydrogen bonds make predominant contribution to the specificity of molecular recognition. The hydrogen attached to nitrogen in the pyrazole ring was H-bonded to the oxygen of the carboxyl group of Asparagine 327 (ASN 327). A second H-bonded interaction was observed between the fluorine attached to the phenyl group of thiadiazepine ring and the hydrogen attached to the nitrogen of Glutamine 457 (GLN 457) residue. The hydrogen bonds made by fluorine are strong enough to contribute to the binding of fluoro compounds to the active site of the receptor. The physicochemical properties of **4e** such as molecular weight (528 Dalton), logP value (6), number of hydrogen acceptors (6) and donors (2) were in compliance with Lipinski's rule (Ro5) for orally active drugs. The number of rotatable bonds was found to be six, which is important for conformation changes of molecule and ultimately for efficient binding with the target protein. Polar surface area (PSA), the sum of vanderwaals surface areas of polar atoms calculated for **4e** was 75.926 Å² which indicated its better oral absorption, intestinal permeability and passive transport.

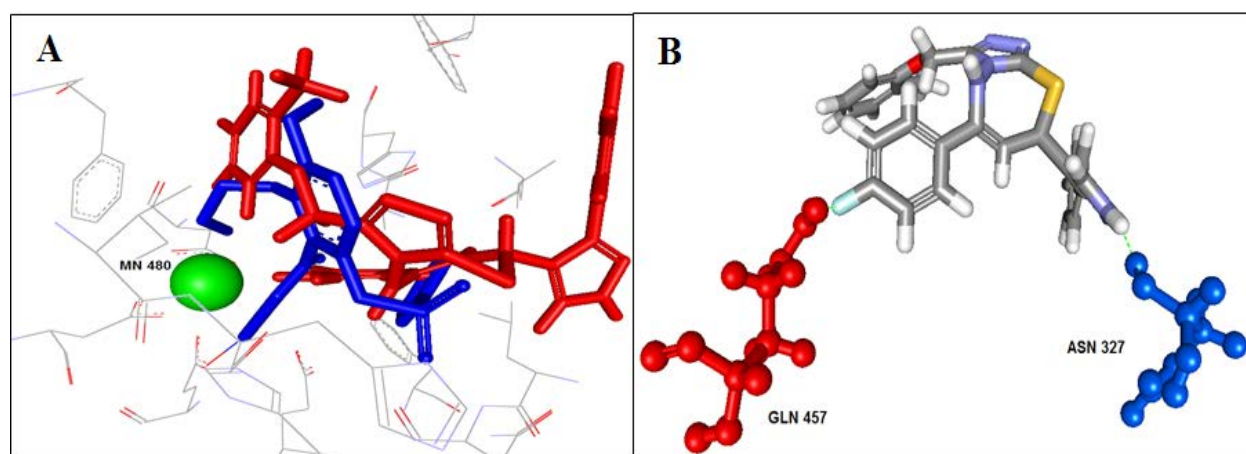


Fig.3 (A) Docked conformers of **4e** (coloured red) and A797859 (coloured blue) (B) Interaction of **4e** with active site residues in MetAP2

In DPPH and ABTS assay, neither the two chalcones nor the thiadiazepines were as potent as ascorbic acid. Chalcone **3a** showed better antioxidant behaviour with low IC_{50} values in ABTS assay, presumably through electron transfer mechanism. Antioxidant potency through hydrogen transfer was observed for **4e** as its IC_{50} value in DPPH assay was lower than that in ABTS assay. The results of DPPH and ABTS assays are depicted in Table-2.

Table 2: *In vitro* antioxidant activity of chalcones and thiadiazepines

Comp. No.	IC_{50} (μ g/mL)	
	DPPH	ABTS
AA	21.50 $\pm$ 1.26	25.33 $\pm$ 1.42
3a	87.15 $\pm$ 2.70	56.45 $\pm$ 3.49
3b	101.48 $\pm$ 7.07	77.44 $\pm$ 6.43
4a	107.28 $\pm$ 8.82	173.75 $\pm$ 5.70
4b	121.72 $\pm$ 11.01	230.89 $\pm$ 3.37
4e	61.26 $\pm$ 3.84	500.72 $\pm$ 8.66
4h	>1000	>1000

Values are mean $\pm$ S.E.M of triplicates, AA: Ascorbic acid

Conclusion

In this study a series of two new pyrazole chalcones and ten new triazolo-thiadiazepines were synthesized. Chalcones were prepared by base catalyzed Claisen-Schmidt condensation of 3-substituted-1*H*-pyrazole carbaldehydes with 4-fluoro acetophenone. Thiadiazepines were synthesized by the reaction between chalcones and triazoles with sodium acetate as catalyst. The different spectral techniques and the elemental analysis confirmed the structure of the compounds. In DPPH and ABTS assays, none of the chalcones or thiadiazepines was as potent as ascorbic acid. **3a** and **4e** was found to show moderate antioxidant properties. This study reveals 3-[(2-methylphenoxy)methyl]-

6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4] triazolo[3,4-*b*] [1,3,4]thiadiazepine to be a potent cytotoxic apoptotic and antiangiogenic agent than currently used anticancer agent doxorubicin against breast adenocarcinoma cells. It also acts as a novel MetAP2 inhibitor and there is ample scope for further study in this area.

Acknowledgements

A.M.I is thankful to the Director, NITK-Surathkal, India for rendering research facilities. D.S is grateful to Director and Head-Department of Chemistry, Manipal Institute of Technology, Manipal University for providing us the necessary laboratory facilities. The authors are thankful to the Head-SIF and Organic Chemistry Department, Indian Institute of Science, Bangalore for providing NMR and mass spectral data respectively.

Experimental

General method for the synthesis of (2*E*)-1-(4-fluorophenyl)-3-[(3-substituted)-1*H*-pyrazol-4-yl]prop-2-en-1-one **3a-b**

1 mmol of 3-substituted-1*H*-pyrazole-4-carbaldehyde **1** in ethanol was added to 10 %, 2 mL sodium hydroxide maintained at 20 °C. 1 mmol of 4-fluoro acetophenone was added to the above mixture, stirred at room temperature for 2 h and kept overnight. The reaction mixture was poured over crushed ice. The yellow solid obtained was filtered and recrystallized from ethanol.

(2*E*)-1-(4-fluorophenyl)-3-[3-(4-fluorophenyl)-1*H*-pyrazol-4-yl]prop-2-en-1-one (**3a**)

Yellow solid (68 %); m.p. 158-160 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H *str.*), 3124 (Ar. C-H *str.*), 1678 (C=O *str.*), 1230 (CH=CH *str.*); ^1H -NMR (DMSO- d_6) [ppm]: δ 7.39 (s, 1H, Pyrazole 5-H), δ 7.64 (d, 1H, J = 15.4 Hz), δ 7.74 (d, 1H, J = 15.4 Hz), δ 7.35-8.17 (8H, Ar.H), δ 8.49 (1H, Pyrazole N-H); MS (m/z):

310 (M^+); Anal. calcd. for $C_{18}H_{12}F_2N_2O$; C, 69.67; H, 3.87; N, 9.03. Found: C, 69.79; H, 3.89; N, 9.08.

(2*E*)-1-(4-fluorophenyl)-3-[3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl]prop-2-en-1-one (**3b**)

Yellow solid (75 %); m.p. 162-164 °C, IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3120 (Ar. C-H str.), 2955, 2830 (methyl C-H str.), 1670 (C=O str.), 1227 (CH=CH str.); ^1H -NMR (DMSO- d_6) [ppm]: δ 3.5 (3H, OCH_3), δ 7.38 (s, 1H, Pyrazole 5-H), δ 7.58 (d, 1H, $J = 15.8$ Hz), δ 7.68 (d, 1H, $J = 15.8$ Hz), δ 7.36-8.2 (8H, Ar.H), 8.42 (1H, Pyrazole N-H); MS (m/z): 322 (M^+); Anal. calcd. for $C_{19}H_{15}FN_2O_2$; C, 70.81; H, 4.66; N, 8.79. Found: C, 71.02; H, 4.67; N, 8.83.

General method for the preparation of 3-[(substituted)methyl]-6-(4-fluorophenyl)-8-(3-substituted-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepines **4a-j**

1 mmol of 5-substituted 4-amino-3-mercapto-1,2,4-triazoles **1** and 1 mmol of chalcones **3** in 15 mL DMSO containing catalytic amount of sodium acetate was refluxed on a water bath for 12 h. Excess solvent was removed and the reaction mixture was kept aside overnight. The solid separated was collected, washed with water, dried and recrystallized from ethanol.

3-(phenoxymethyl)-6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4a**)

Yellow solid (78 %); m.p. 238-240 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3122 (Ar. C-H str.), 1305 (C-N str.), 1240 (C-O-C asym. str.), 1040 (C-O-C sym. str.); ^1H -NMR (DMSO- d_6) [ppm]: δ 5.02 (d, >CH-S of thiadiazepine ring), δ 5.45 (s, 2H, OCH_2), δ 7.61 (d, -CH= of thiadiazepine ring), δ 7.66 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 13H, Ar.H), δ 8.49 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 514 (M^+); Anal. calcd. for $C_{27}H_{20}F_2N_6OS$; C, 63.04; H, 3.89; N, 16.34. Found: C, 63.19; H,

3.91; N, 16.41.

3-(phenoxymethyl)-6-(4-fluorophenyl)-8-(3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4b**)

Yellow solid (81 %); m.p. 230-232 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3124 (Ar. C-H str.), 2930, 2840 (methyl C-H str.), 1308 (C-N str.), 1242 (C-O-C asym. str.), 1031 (C-O-C sym. str.); ^1H -NMR (DMSO- d_6) [ppm]: δ 3.82 (s, 3H, OCH_3), δ 5.02 (d, >CH-S of thiadiazepine ring), δ 5.43 (s, 2H, OCH_2), δ 7.58 (d, -CH= of thiadiazepine ring), δ 7.61 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 13H, Ar.H), δ 8.41 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 526 (M^+) (Fig. 6.18); Anal. calcd. for $C_{28}H_{23}FN_6O_2S$; C, 63.88; H, 4.37; N, 15.97. Found: C, 64.01; H, 4.40; N, 16.02.

3-[(4-methylphenoxy)methyl]-6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4c**)

Yellow solid (76 %); m.p. 224-226 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3120 (Ar. C-H str.), 2940, 2850 (methyl C-H str.), 1307 (C-N str.), 1245 (C-O-C asym. str.), 1031 (C-O-C sym. str.); ^1H -NMR (DMSO- d_6) [ppm]: δ 2.39 (s, 3H, CH_3), δ 5.03 (d, >CH-S of thiadiazepine ring), δ 5.43 (s, 2H, OCH_2), δ 7.57 (d, -CH= of thiadiazepine ring), δ 7.62 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 12H, Ar.H), δ 8.43 (1H, Pyrazole N-H), 8.50 (1H, thiadiazepine N-H); MS (m/z): 528 (M^+); Anal. calcd. for $C_{28}H_{22}F_2N_6OS$; C, 63.64; H, 4.17; N, 15.91. Found: C, 63.79; H, 4.19; N, 16.95.

3-[(4-methylphenoxy)methyl]-6-(4-fluorophenyl)-8-(3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4d**)

Yellow solid (79 %); m.p. 235-237 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3120 (Ar. C-H str.), 2935, 2840 (methyl C-H str.), 1308 (C-N str.), 1240 (C-O-C asym. str.), 1030 (C-O-C

sym. str.); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 2.4 (s, 3H, CH_3), δ 3.83 (s, 3H, OCH_3), δ 5.03 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.42 (s, 2H, OCH_2), δ 7.55 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.63 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 12H, Ar.H), δ 8.42 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 540 (M^+); Anal. calcd. for $\text{C}_{29}\text{H}_{25}\text{FN}_6\text{O}_2\text{S}$; C, 64.44; H, 4.63; N, 15.56. Found: C, 64.61; H, 4.65; N, 15.60.

3-[(2-methylphenoxy)methyl]-6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4e**)

Yellow solid (85 %); m.p. 218-220 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3119 (Ar. C-H str.), 2940, 2850 (methyl C-H str.), 1308 (C-N str.), 1240 (C-O-C asym. str.), 1035 (C-O-C sym. str.); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 2.39 (s, 3H, CH_3), δ 5.02 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.42 (s, 2H, OCH_2), δ 7.54 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.65 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 12H, Ar.H), δ 8.45 (1H, Pyrazole N-H), 8.52 (1H, thiadiazepine N-H); MS (m/z): 528 (M^+); Anal. calcd. for $\text{C}_{28}\text{H}_{22}\text{F}_2\text{N}_6\text{OS}$; C, 63.64; H, 4.17; N, 15.91. Found: C, 63.75; H, 4.20; N, 15.98.

3-[(2-methylphenoxy)methyl]-6-(4-fluorophenyl)-8-(3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4f**)

Yellow solid (82 %); m.p. 222-224 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3120 (Ar. C-H str.), 2935, 2840 (methyl C-H str.), 1308 (C-N str.), 1242 (C-O-C asym. str.), 1035 (C-O-C sym. str.); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 2.4 (s, 3H, CH_3), δ 3.83 (s, 3H, OCH_3), δ 5.02 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.41 (s, 2H, OCH_2), δ 7.55 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.65 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 12H, Ar.H), δ 8.41 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 540 (M^+); Anal. calcd. for $\text{C}_{29}\text{H}_{25}\text{FN}_6\text{O}_2\text{S}$; C, 64.44; H, 4.63; N, 15.56. Found: C, 64.72; H, 4.65; N, 15.59.

3-(1-naphthyloxymethyl)-6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4g**)

Dark yellow solid (79 %); m.p. 198-200 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3120 (Ar. C-H str.), 1302 (C-N str.), 1249 (C-O-C asym. str.), 1040 (C-O-C sym. str.), 825 (Ar. C-H def.), 735, 822, 842 (naphthalene C-H def.); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 5.03 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.41 (s, 2H, OCH_2), δ 7.56 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.64 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 15H, Ar.H), δ 8.43 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 564 (M^+); Anal. calcd. for $\text{C}_{31}\text{H}_{22}\text{F}_2\text{N}_6\text{OS}$; C, 65.95; H, 3.90; N, 14.89. Found: C, 66.09; H, 3.94; N, 14.93.

3-(1-naphthyloxymethyl)-6-(4-fluorophenyl)-8-(3-(4-methoxyphenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4h**)

Yellow solid (83 %); m.p. 214-216 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3120 (Ar. C-H str.), 2940, 2850 (methyl C-H str.), 1308 (C-N str.), 1250 (C-O-C asym. str.), 1035 (C-O-C sym. str.), 825 (Ar. C-H def.), 732, 820, 842 (naphthalene C-H def.); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 3.82 (s, 3H, OCH_3), δ 5.02 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.41 (s, 2H, OCH_2), δ 7.57 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.65 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 15H, Ar.H), δ 8.45 (1H, Pyrazole N-H), 8.52 (1H, thiadiazepine N-H); MS (m/z): 576 (M^+); Anal. calcd. for $\text{C}_{32}\text{H}_{25}\text{FN}_6\text{O}_2\text{S}$; C, 66.67; H, 4.34; N, 14.58. Found: C, 66.83; H, 4.37; N, 14.61.

3-(2-naphthyloxymethyl)-6-(4-fluorophenyl)-8-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-5,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4i**)

Dark yellow solid (75 %); m.p. 223-225 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H str.), 3125 (Ar. C-H str.), 1302 (C-N str.), 1247 (C-O-C asym. str.), 1040 (C-O-C sym. str.), 828 (Ar. C-H def.), 730, 820, 840 (naphthalene C-H

def.); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 5.02 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.42 (s, 2H, OCH_2), δ 7.56 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.66 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 15H, Ar.H), δ 8.43 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 564 (M^+); Anal. calcd. for $\text{C}_{31}\text{H}_{22}\text{F}_2\text{N}_6\text{OS}$; C, 65.95; H, 3.90; N, 14.89. Found: C, 66.16; H, 3.91; N, 14.91.

3-(2-naphthyloxymethyl)-6-(4-fluorophenyl)-8-(1*H*-pyrazol-4-(4-methoxyphenyl))-7,8-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazepine (**4j**)

Yellow solid (73 %); m.p. 218-220 °C; IR (KBr) [cm^{-1}]: 3400 (pyrazole N-H *str.*), 3119 (Ar. C-H *str.*), 2930, 2840 (methyl C-H *str.*), 1308 (C-N *str.*), 1245 (C-O-C *asym. str.*), 1038 (C-O-C *sym. str.*), 825 (Ar. C-H *def.*), 735, 820, 840 (naphthalene C-H *def.*); $^1\text{H-NMR}$ (DMSO-d_6) [ppm]: δ 3.83 (s, 3H, OCH_3), δ 5.02 (d, $>\text{CH-S}$ of thiadiazepine ring), δ 5.42 (s, 2H, OCH_2), δ 7.55 (d, $-\text{CH=}$ of thiadiazepine ring), δ 7.65 (s, 1H, pyrazole 5-H), δ 6.9-8.2 (m, 15H, Ar.H), δ 8.42 (1H, Pyrazole N-H), 8.51 (1H, thiadiazepine N-H); MS (m/z): 576 (M^+); Anal. calcd. for $\text{C}_{32}\text{H}_{25}\text{FN}_6\text{O}_2\text{S}$; C, 66.67; H, 4.34; N, 14.58. Found: C, 66.83; H, 4.36; N, 14.62.

References

1. T.K. Lee, T.C. Lau, O.N. Irene, Cancer Chemother. Pharmacol., **2002**, 49, 78-86.
2. S.Q. Yin, J.J. Wang, C.M. Zhang, Z.P. Liu, Curr. Med. Chem., **2012**, 19(7), 1021-1035.
3. A. Ghosh, R. Datta, A. Majumdar, M. Bhattacharya, B. Datta, Exp. Cell. Res., **2006**, 312, 3184-3203.
4. A.L. Tucker, Q. Zhang, G.S. Sheppard, P. Lou, F. Jiang, E. McKeegan, R. Lesniewski, S.K. Davidsen, R.L. Bell, J. Wang, Oncogene, **2008**, 27, 3967-3976.
5. C.H.M. Alvin, T.K. Fung, R.H.C. Lin, M.I.S. Chung, D. Yang, S.C. Ekker, A.Y.H. Leung, Blood, **2011**, 118 (20), 5448-5457.
6. P. Selvakumar, A. Lakshmikuttyamma, J.R. Dimmock, R.K. Sharma, Biochim. Biophys. Acta, **2006**, 1765(2), 148-154.
7. B. Datta, R. Datta, Exp. Cell Res., **1999**, 246, 376-383.
8. S.G. Bernier, N. Taghizadeh, C.D. Thompson, W.F. Westlin, G. Hannig, J. cell Biochem., **2005**, 95, 1191-1203.
9. S. Kidoikhamouan, W. Seubwai, N. Tantapotin, A. Silsirivanit, S. Wongkham, K. Sawanyawisuth, C. Wongkham, Asian Pac. J. Cancer Prev., **2012**, 13, 155-160.
10. H. Bo, M. Ghazizadeh, H. Shimizu, Y. Kurihara, S. Egawa, Y. Moriyama, T. Tajiri, O.J. Kawanami, Nippon Med. Sch., **2004**, 71(3), 172-180.
11. H. Zhang, J.J. Liu, J. Sun, X.H. Yang, T.T. Zhao, X. Lu, H.B. Gong, H.L. Zhu, Bioorg. Med. Chem., **2012**, 20(10), 3212-3218.
12. C.T. Hsieh, T.J. Hsieh, M. El-Shazly, D.W. Chuang, Y.H. Tsai, C.T. Yen, S.F. Wu, Y.C. Wu, F.R. Chang, Bioorg. Med. Chem. Lett., **2012**, 22(12), 3912-3915.
13. K.V. Sashidhara, S.R. Avula, G.R. Palnati, S.V. Singh, K. Srivastava, S.K. Puri, J.K. Saxena, Bioorg. Med. Chem. Lett., **2012**, 22(17), 5455-5459.
14. R. Dandia, S. Singh, Khaturia, Bioorg. Med. Chem., **2006**, 14(5), 1303-1308.
15. B. Kalluraya, P. Vishwanatha, C.H. Jyothi, V.F. Priya, G. Rai, Indian J. Het. Chem., **2003**, 12, 355-356.
16. A.R. Farghaly, E. De Clercq, H. El-Kashef, Arkivoc, **2006**, 10, 137-151.
17. J.F. Ramírez-Martínez, R. González-Chávez, R. Guerrero-Alba, P.E. Reyes-Gutiérrez, R. Martínez, M. Miranda-Morales, R. Espinosa-Luna, M.M. González-Chávez, C. Barajas-López, Molecules, **2013**, 18, 894-913.
18. G. Monica, P. Satya, G. Rajive, Indian J. Heterocycl. Chem., **2009**, 48B, 460-466.
19. F.R. Juan, G. Rodolfo, G. Raquel, E.R. Paul, M. Roberto, M. Marcelo, E. Rosa, M.G. Marco, B. Carlos, Molecules, **2013**, 18, 894-913.
20. P. Om, S. Deepak, K. Ravi, Indian J. Heterocycl. Chem., **2004**, 14, 111-114.
21. M. Kidwai, P. Sapra, R.K. Saxena, M. Singh, Bioorg. Med. Chem., **2001**, 9, 217-220.
22. R.E. Iraj, C. Charalabos, Z. Panagiotis, G. Athina, S. Marina, G. Jasmina, C. Ana, Bioorg. Med. Chem., **2008**, 16, 1150-1161.
23. A.K. Zafer, T. Gülhan, Ö. Ahmet, R. Gilbert, Eur. J. Med. Chem., **2008**, 43, 155-159.
24. V.S. Palekar, A.J. Damle, S.R. Shukla, Eur. J. Med. Chem., **2009**, 44, 5112-5116.
25. D.A. Ibrahim, Eur. J. Med. Chem., **2009**, 44, 2776-2781.
26. M. Gupta, S. Paul, R. Gupta, Indian J. Chem., **2009**, 48B, 460-466.
27. W. Robert, Brueggemeier, C.H. John, S.D. Edgar, Endocrine Rev., **2004**, 26, 331-345.
28. C. Alka, P.K. Sharma, K. Niranjana, Intl. J. Chem. Tech. Res., **2011**, 3, 11-17.
29. A.M. Vijesh, A.M. Isloor, V. Prabhu, S. Ahmad, S. Malladi, Eur. J. Med. Chem., **2010**, 45, 5460-5464.
30. A.M. Vijesh, A.M. Isloor, S. Telkar, S.K. Peethambar, S. Rai, N. Isloor, Eur. J. Med. Chem., **2011**, 46, 3531-3536.
31. D. Sunil, A.M. Isloor, P. Shetty, K. Satyamoorthy, A.S. Bharathprasad, Arabian J. Chem., **2010**, 3, 211-217.
32. R.N. Gacche, N.A. Dhole, S.G. Kamble, B.P. Bandgar, J.

- Enzym. Inhib. Med. Chem., **2008**, 23, 28-31.
33. P.B. Babasaheb, S.G. Shrikant, G.B. Ragini, V.T. Jalinde, N.K. Chandrahas, Bioorg. Med. Chem., **2010**, 18, 1364-1370.
 34. B. Chandrakantha, S. Prakash, N. Vijesh, I. Nishitha, A.M. Isloor, Eur. J. Med. Chem., **2010**, 45(3), 1206-1210.
 35. A.M. Vijesh, A.M. Isloor, P. Shetty, S. Sundershan, K.F. Hoong, Eur. J. Med. Chem. **2013**, 62, 410-415.
 36. A.M. Vijesh, A.M. Isloor, S. Telkar, T. Arulmoli, Arabian J. Chem., **2013**, 6(2), 197-204.
 37. D. Sunil, A.M. Isloor, P. Shetty, B. Chandrakantha, K. Satyamoorthy, Med. Chem. Res., **2011**, 20, 1024-1032.
 38. A.M. Isloor, B. Kalluraya, P. Shetty, Eur. J. Med. Chem., **2009**, 44, 3784-3787.
 39. D. Dunil, A.M. Isloor, P. Shetty, K. Satyamoorthy, A.S. Bharathprasad, Med. Chem. Res., **2011**, 20, 1074-1080.
 40. A.M. Isloor, S. Dhanya, P. Shetty, M. Shridhar, K.S.R.; Pai, M. Naseer, Med. Chem. Res., **2013**, 22, 758-767.
 41. S.N. Manjula, M. Kenganora, V.K. Parihar, S. Kumar, P.G. Nayak, N. Kumar, K.S.R. Pai, C.M. Rao, Pharm. Biol., **2010**, 48(6), 690-696.
 42. V.P. Veerapur, K.R. Prabhakar, V.K. Parihar, M.R. Kandadi, S. Ramakrishana, B. Mishra, B.S.S. Rao, K.K. Srinivasan, K.I. Priyadarsini, M.K. Unnikrishnan, Alternat. Med., **2009**, 6(3), 317-324.