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Original article

Synthesis and evaluation of 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles as anti-cancer agents

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ABSTRACT

A series of 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles **9a**–**j** were obtained via multistep synthesis from hydroxybenzophenones **4a**–**e**. The cytotoxicity of compounds **9a**–**j** was evaluated against human leukemia cell lines (K562 and CEM). The compounds exhibited moderate to good anti-cancer activity with compounds **9b** and **9i** having a chloro group exhibiting the best activity (IC_{50} = 10 μM). Compound **9i** exhibited activity against both the cell lines and **9b** only exhibited activity against CEM. Further, a lactate dehydrogenase (LDH) assay and DNA fragmentation studies of the compounds **9a**–**j** were also performed.

1. Introduction

Cancer is a disease in which a group of cells display uncontrolled growth in a body. Under normal conditions, cell death and cell proliferation are balanced throughout the life of multicellular organisms [1]. The various types of mature cells in the body have a specific life span. After the death of these cells, new cells are generated by proliferation and differentiation into different types of cells. Sometimes cells begin to divide in an uncontrolled manner and they no longer respond to the growth regulatory mechanisms. These cells divide and form a clump of cells producing a tumor or neoplasm [2]. About 200 types of cancers affecting major organs like lungs, brain, kidneys, colon, breasts and stomach have been identified. According to the National Cancer Institute, most can fit into categories of carcinoma, sarcoma, leukemia, lymphoma, and central nervous system cancers. The development of efficient, selective and less toxic anti-cancer agents is a challenge in cancer research [3].

Studies have shown that modification in DNA and RNA through mutation leads to altered protein production which is the main cause for the development of cancer [4–7]. Nowadays the field of drug discovery for cancer has expanded in such a way as to target the disease in regulating DNA replication by using nucleotide analogs like 5-fluorouracil and enzyme inhibitors like methotrexate [8,9]. Other than that imatinib, a drug developed against the activated tyrosine kinases in chronic myelogenous leukemia is yet another development in the field of cancer [10].

Heterocyclic compounds play an important role as anti-cancer agents because of their excellent inhibitory activity against receptor tyrosine kinases [11], raf kinases [12], protein tyrosine kinases [13] and NADH oxidase [14], which play critical roles in many aspects of tumorigenesis. Among heterocyclic compounds, nitrogen containing compounds like 1,3,4 oxadiazoles have exhibited broad spectrum anti-tumor activity with an IC_{50} concentration range from 7.21 μM to 25.87 μM against the HeLa cancer cell lines [15] and with an IC_{50} of 9.3 μM against the DU145 cancer cell lines [16]. Oxadiazole analogs also inhibit bacterial growth by inhibiting DNA replication or DNA transcription [17].

In continuation of our research work on anti-cancer agents [18,19] and with the goal of discovering new anti-cancer agents, we have focused on the synthesis of a novel 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles **9a**–**j** which possess anti-proliferative property by inhibiting cell cycle and promoting apoptosis.

2. Chemistry

The synthesis of the hitherto unreported title compounds is as outlined in Scheme 1. (4-Hydroxyaryl)aryl methanones commonly

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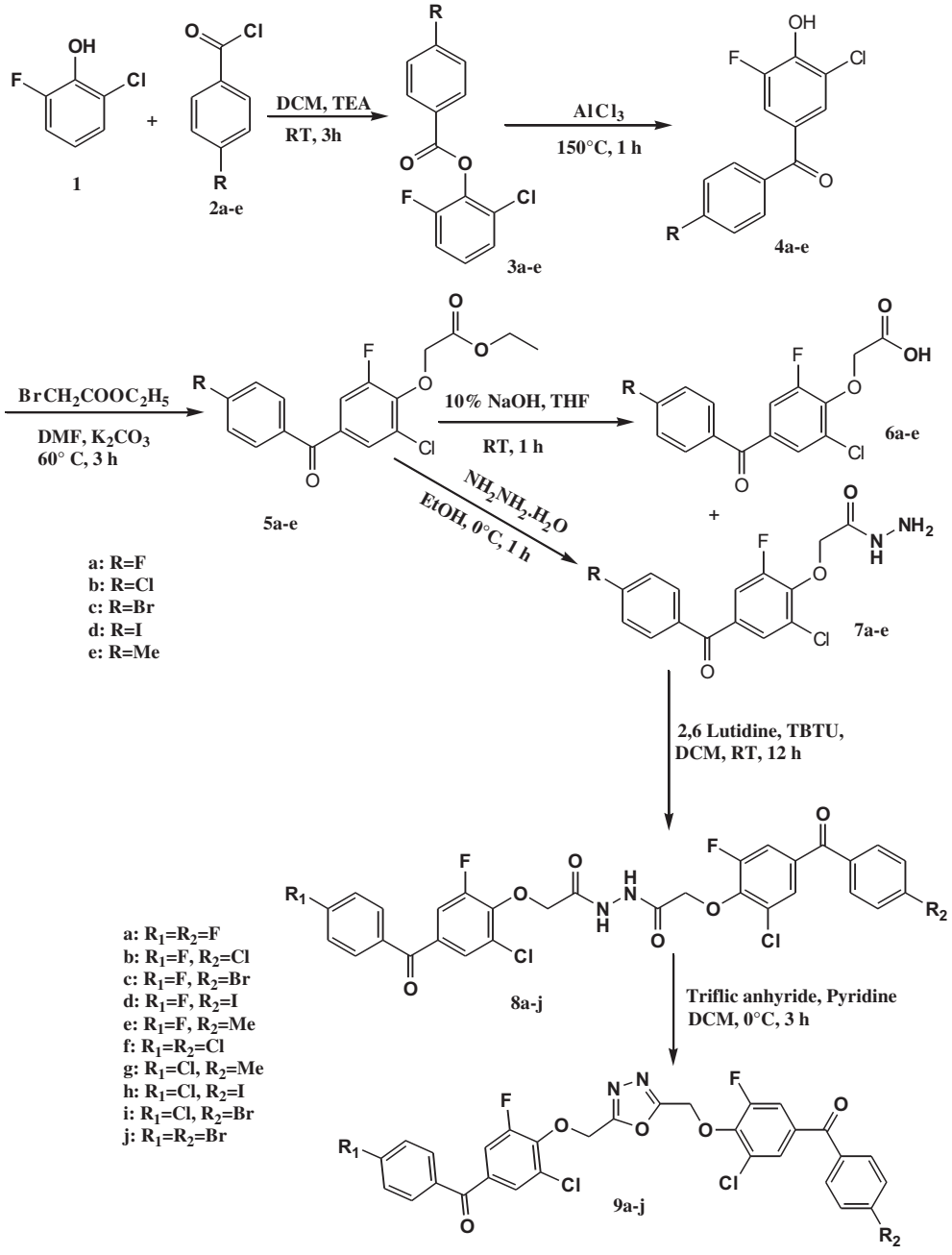
known as hydroxybenzophenones **4a**–**e** were achieved in excellent yield using benzoylation of compound **1** with benzoyl chloride derivatives **2a**–**e** followed by Fries rearrangement of substituted arylbenzoates **3a**–**e**. Compounds **4a**–**e** on reaction with ethyl bromoacetate afforded ethyl 4-aryloylaryloxyacetates **5a**–**e** which on treatment with sodium hydroxide in presence of THF gave 4-aryloylaryloxyethanoic acids **6a**–**e**. Further, compounds **5a**–**e** on treatment with hydrazine hydrate in the presence of ethanol yield 4-aryloylaryloxyacethydrazides **7a**–**e**. Condensation of **6a**–**e** with **7a**–**e** in the presence of 2,6 lutidine, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and dichloromethane (DCM) afforded *N*,*N*-di(2-(4-aryloylaryloxy) acetyl)hydrazines **8a**–**j**. Finally, title compounds **9a**–**j** were achieved by intramolecular cyclization of **8a**–**j** in the presence of triflic anhydride, pyridine and DCM.

3. Pharmacology

The human leukemia cells, K562 and CEM were selected for the purpose of preliminary anti-cancer screening of newly synthesized compounds **9a**–**j**. To assess the cytotoxicity, trypan blue dye exclusion assay, MTT assay and LDH assay were employed. For this, cells growing in log phase were treated with different concentrations (10, 50, 100 and 250 μM) of the title compounds **9a**–**j**. Besides, cytotoxicity of compounds **9a**–**j** on the growth of normal cells was assessed using MTT assay.



Scheme 1. Synthesis of 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles.

4. Results and discussion

4.1. Chemistry

1,3,4-Oxadiazole ring in the title compounds **9a**–**j** was constructed by cyclizing *N*,*N*-di(2-(4-aryloylaryloxy)acetyl)hydrazines **8a**–**j** in the presence of pyridine and triflic anhydride in DCM. The structures of the compounds were elucidated by IR, ^{1}H NMR and mass spectral studies and microanalyses.

4.2. Biology

Newly synthesized 1,3,4-oxadiazole analogs **9a**–**j** were assessed for cytotoxicity against two human leukemia cell lines, K562 (Chronic myelogenous leukemia) and CEM (T-cell leukemia). The effective concentrations of compounds **9a**–**j** required to inhibit K562 and CEM cell growth and survival were determined by carrying out dose response experiments using a trypan blue dye exclusion assay. The cell viability was further assessed by MTT assay and 5-fluorouracil treated cells were used as a positive control. The cells with DMSO (equivalent to DMSO used in 250 μM) were used as vehicle control, since the compounds **9a**–**j** were dissolved in DMSO. The number of viable cells decreased at different points of time and concentrations on exposure to compounds **9a**–**j** (Fig. 1). Compounds **9a**–**j** did not exhibit any significant inhibition of cell proliferation at a concentration of 10 μM against both cell lines. The cell viability at both 48 and 72 h were affected at 50 and 100 μM concentrations for compounds **9b**–**f** and **9h**–**j**. Compounds **9b** with fluoro and chloro groups, **9e** with fluoro and methyl groups, **9i** with chloro and bromo groups and **9j** with two bromo groups in the two different benzophenone moieties showed complete inhibition at 48 and 72 h treatment at a 250 μM concentration against the CEM cell line (Fig. 1). Compound **9i** was most effective at 50 μM exhibiting complete inhibition. However the DMSO control did not show any significant toxic effect. Compounds **9a** with two fluoro groups in the two different benzophenone moieties and **9g** with chloro and methyl groups in the two different benzophenone moieties showed moderate inhibitory activity. The electron withdrawing halo groups at the para position in the benzophenone moieties are important for enhancing the inhibitory activity while the electron releasing methyl group at the para position decreases the activity as seen in **9e** and **9g** (Table 1).

Release of LDH serves as a marker for membrane integrity. Hence, LDH release assay was performed to test the cell damage by compounds **9b**, **9e**, **9i** and **9j**. Results showed a dose- and time-dependent increase in LDH (Fig. 1), which further confirmed our results.

Oligonucleosomal DNA fragmentation and nuclear condensation are the criteria to analyze the DNA damage consequent upon treatment with compounds **9b** and **9i**. From the K562 cells treated with increasing concentrations of compounds **9b** and **9i** chromosomal DNA was extracted and used for agarose gel electrophoresis. The result of this assay showed that DNA fragmentation leads to a smear formation in gel lanes in which cells treated with compounds **9b** and **9i** (Fig. 2). Number of breaks in the chromosomal DNA of K562 cells resulted in smearing. The intensity of smear increased with the dose, 50 μM showing moderate and 100 μM showing maximum smearing (Fig. 2). This suggests that compounds **9b** and **9i** induce fragmentation of chromosomal DNA leading to apoptosis.

Table 1
IC_{50} values of compounds 9a–j as determined based on MTT assay.

Compound	IC_{50} (μM)	
	K562	CEM
9a	190 ± 10.0	195 ± 12.2
9b	16 ± 2.8	10 ± 3.5
9c	60 ± 5.5	62 ± 6.1
9d	75 ± 7.2	78 ± 7.6
9e	22 ± 4.4	24 ± 4.8
9f	73 ± 7.0	75 ± 7.2
9g	122 ± 10.0	125 ± 10.4
9h	73 ± 6.0	74 ± 6.5
9i	10 ± 2.1	12 ± 2.2
9j	20 ± 2.8	20 ± 3.0
5-Fluorouracil	28 ± 3.8	32 ± 4.1

5. Conclusion

In summary, a series of 1,3,4 oxadiazoles **9a**–**j** were synthesized and evaluated for anti-proliferative activity against human leukemic cell lines. From the current investigation, structural activity relationship of these compounds suggests that the position and the type of substituent on the aromatic ring in **9a**–**j** are important for activity. Compounds **9b** and **9i** with chloro group play a dominant role in inhibiting the leukemic cell proliferation. Further, a detailed investigation on the structural activity relationship should entail structural modification on the aromatic ring for the discovery of more potent cytotoxic compounds. Studies on the mechanism of action of the title compounds and modification are under progress.

6. Experimental section

Chemicals were purchased from Sigma Aldrich Chemical Co. TLC was performed on aluminum-backed silica plates and visualized by UV-light. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded on FT-IR Shimadzu 8300 spectrophotometer, ^{1}H NMR spectra were recorded on a Bruker 400 MHz NMR

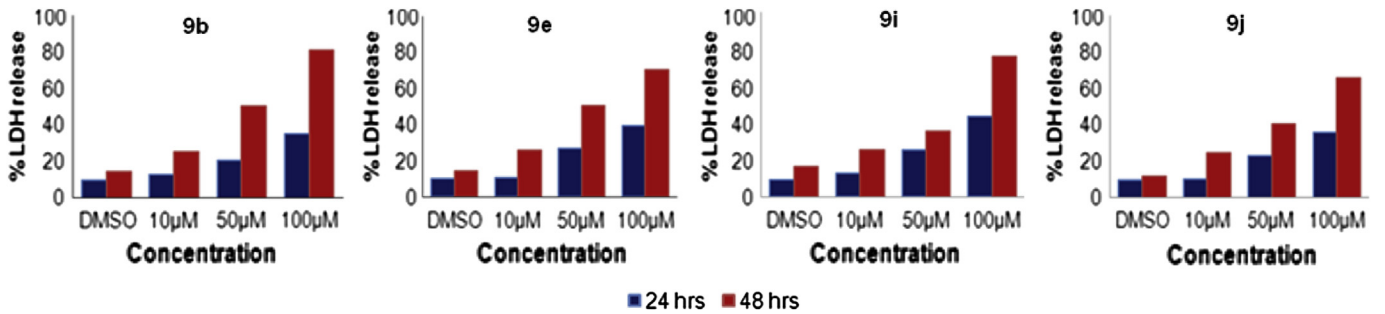


Fig. 1. Time-and dose dependent LDH release in K562 cells treated with compounds **9b**, **9e**, **9i** and **9j**. Cells were incubated for 24 and 48 h with different concentrations of compounds **9b**, **9e**, **9i** and **9j**. Release of LDH in the medium was measured at 490 nm. Results are presented as percentage of LDH release.

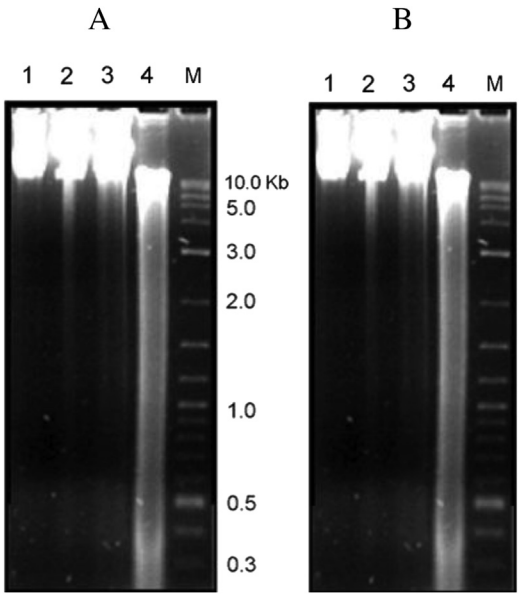


Fig. 2. Detection of DNA damage induced by 9ior 9j in K562 cells. The chromosomal DNA was extracted from K562 cells treated with different concentrations of **9i** (A) and **9j** (B). The purified DNA was then resolved on a 1% agarose gel at 30 V for 6 h. In both panels, lane 1: DMSO; lane 2–4: K562 cells treated with 10, 50 and 100 μM, respectively. "M" is marker.

spectrophotometer in DMSO-d_6 and the chemical shifts were recorded in parts per million down field from tetramethylsilane. Mass spectra were obtained with a VG70-70H spectrophotometer and important fragments are given with the relative intensities in brackets. Results of elemental analysis are within 0.4% of the calculated value.

6.1. Synthesis

6.1.1. General procedure for substituted arylbenzoates (**3a**–**e**)

2-Chloro-6-fluoro phenol (**1**, 0.2054 mol) was dissolved in DCM, triethylamine (TEA, 0.4519 mol) was added and the reaction mixture was cooled to 0 °C. A solution of benzoyl chloride derivatives (**2a**–**e**, 0.2157 mol) in DCM was added slowly to the above mixture and stirred for 3 h. Then the reaction mass was diluted with DCM (200 mL), washed with 10% sodium hydroxide solution (3 × 30 mL), water (3 × 30 mL), brine (2 × 60 mL), and again with water (3 × 30 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated to achieve compounds **3a**–**e**.

*6.1.1.1. Synthesis of 2-chloro-6-fluorophenyl-4-fluorobenzoate (**3a**).* 2-Chloro-6-fluoro phenol (**1**, 30 g, 0.2054 mol) was dissolved in DCM, triethylamine (TEA, 45.73 g, 0.4519 mol) was added and the reaction mixture was cooled to 0 °C. A solution of 4-fluorobenzoyl chloride (**2a**, 33.9 g, 0.2157 mol) in DCM was added slowly to the above mixture and internal temperature was maintained to 0–10 °C. Finally the reaction mixture was stirred at ambient temperature for 3 h. Then the reaction mass was diluted with DCM (200 mL), washed with 10% sodium hydroxide solution (3 × 30 mL), water (3 × 30 mL), brine (2 × 60 mL), and again with water (3 × 30 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated to achieve compound **3a** as white solid. Yield: 94%; m.p.: 52.6–54.1 °C; IR (KBr) ν_{max} (cm^{-1}): 1738 (ester, C=O); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.42–7.53 (m, 4H, Ar–H), 8.25–8.28 (m, 3H, Ar–H); MS (EI): *m/z* (75%) M^+ 268.5; Anal. Calcd. for $C_{13}H_7ClF_2O_2$ (268.5): C, 58.12; H, 2.63; Cl, 13.20; F, 14.14. Found: C, 58.22; H, 2.43; Cl, 13.30; F, 14.29%.

Compounds **3b**–**e** were synthesized analogously starting with **2b**–**e** respectively.

3b: Yield: 95%; m.p.: 52.1–53.5 °C; IR (KBr) ν_{max} (cm^{-1}): 1750 (ester, C=O); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.39–7.51 (m, 3H, Ar–H), 7.69–7.71 (d, 2H, Ar–H), 8.16–8.17 (d, 2H, Ar–H). MS (EI): *m/z* (72%) M^+ 285; Anal. Calcd. for $C_{13}H_7Cl_2FO_2$ (285): C, 54.77; H, 2.47; Cl, 24.87; F, 6.66. Found: C, 54.57; H, 2.33; Cl, 24.64; F, 6.42%.

3c: Yield: 97%; m.p.: 67.2–68.7 °C; IR (KBr) ν_{max} (cm^{-1}): 1710 (ester, C=O); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.40–7.53 (m, 3H, Ar–H), 7.85–7.87 (d, 2H, Ar–H), 8.08–8.10 (d, 2H, Ar–H). MS (EI): *m/z* (70%) M^+ 329.5; Anal. Calcd. for $C_{13}H_7BrClFO_2$ (329.5): C, 47.38; H, 2.14; Br, 24.25; Cl, 10.76; F, 5.76. Found: C, 47.18; H, 2.29; Br, 24.36; Cl, 10.51; F, 5.58%.

3d: Yield: 93%; m.p.: 78.4–79.2 °C; IR (KBr) ν_{max} (cm^{-1}): 1765 (ester, C=O); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.39–7.54 (m, 3H, Ar–H), 7.66–7.97 (d, 2H, Ar–H), 8.03–8.06 (d, 2H, Ar–H). MS (EI): *m/z* (74%) M^+ 376.5; Anal. Calcd. for $C_{13}H_7ClFIO_2$ (376.5): C, 41.47; H, 1.87; Cl, 9.42; F, 5.05; I, 33.70. Found: C, 41.29; H, 1.68; Cl, 9.31; F, 5.23; I, 33.53%.

3e: Yield: 96%; m.p.: 62.0–63.1 °C; IR (KBr) ν_{max} (cm^{-1}): 1780 (ester, C=O); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.38–7.51 (m, 3H, Ar–H), 7.6–7.9 (d, 2H, Ar–H), 8.05–8.07 (d, 2H, Ar–H). MS (EI): *m/z* (78%) M^+ 264.5; Anal. Calcd. for $C_{14}H_{10}ClFO_2$ (264.5): C, 63.53; H, 3.81; Cl, 13.39; F, 7.18. Found: C, 63.69; H, 3.71; Cl, 13.09; F, 7.30%.

6.1.2. General procedure for (4-hydroxyaryl)aryl methanones (**4a**–**e**)

Compound **3a**–**e** (0.1903 mol) and aluminum chloride (0.5388 mol) were blended and the mixture was heated to 150 °C and this temperature was maintained for 1 h. Then the reaction mixture was cooled to 0 °C and quenched with 6 N hydrochloric acid (200 mL) and extracted with DCM (3 × 100 mL). The organic layer was washed with water (3 × 40 mL), brine (3 × 30 mL) and again with water (3 × 40 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford compounds **4a**–**e**.

*6.1.2.1. Synthesis of (3-chloro-5-fluoro-4-hydroxyphenyl)4-fluorophenyl methanone (**4a**).* Compound **3a** (51 g, 0.1903 mol) and aluminum chloride (71.05 g, 0.5388 mol) were blended and the mixture was heated to 150 °C and this temperature was maintained for 1 h. Then the reaction mixture was cooled to 0 °C and quenched with 6 N hydrochloric acid (200 mL) and extracted with DCM (3 × 100 mL). The combined organic layer was washed with water (3 × 40 mL), brine (3 × 30 mL) and again with water (3 × 40 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford compound **4a** as pale yellow solid.

Yield: 61%; m.p.: 146.3–147.7 °C; IR (KBr) ν_{max} (cm^{-1}): 1671 (C=O), 3545–3635 (OH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.36–7.82 (m, 6H, Ar–H), 11.64 (bs, 1H, OH). MS (EI): *m/z* (83%): M^+ 268.5; Anal. Calcd. for $C_{13}H_7ClF_2O_2$ (268.5): C, 58.12; H, 2.63; Cl, 13.20; F, 14.14. Found: C, 58.21; H, 2.52; Cl, 13.20; F, 14.25%.

Compounds **4b**–**e** were synthesized analogously starting with **3b**–**e** respectively.

4b: Yield: 68.6%; m.p.: 167.8–169.1 °C; IR (KBr) ν_{max} (cm^{-1}):1660 (C=O), 3525–3625 (OH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.52–7.94 (m, 6H, Ar–H), 11.60 (bs, 1H, OH). MS (EI): *m/z* (80%): M^+ 285; Anal. Calcd. for $C_{13}H_7Cl_2FO_2$ (285): C, 54.77; H, 2.47; Cl, 24.87; F, 6.66. Found: C, 54.65; H, 2.32; Cl, 24.711; F, 6.53%.

4c: Yield: 71%; m.p.: 172.1–173.3 °C; IR (KBr) ν_{max} (cm^{-1}): 1610 (C=O), 3495–3580 (OH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.54–7.79 (m, 6H, Ar–H), 11.63 (bs, 1H, OH). MS (EI): *m/z* (81%): M^+ 329.5; Anal. Calcd. for $C_{13}H_7BrClFO_2$ (329.5): C, 47.38; H, 2.14; Br,

24.25; Cl, 10.76; F, 5.76. Found: C, 47.46; H, 2.29; Br, 24.41; Cl, 10.59; F, 5.62%.

4d: Yield: 65%; m.p.: 182.1–183.2 °C; IR (KBr) ν_{max} (cm^{-1}): 1635 (C=O), 3430–3590 (OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 7.46–7.8 (m, 6H, Ar–H), 11.60 (bs, 1H, OH). MS (EI): *m/z* (79%): M^+ 376.5; Anal. Calcd. for $C_{13}H_7ClFIO_2$ (376.5): C, 41.47; H, 1.87; Cl, 9.42; F, 5.05; I, 33.70. Found: C, 41.32; H, 1.71; Cl, 9.58; F, 5.21; I, 33.61%.

4e: Yield: 68%; m.p.: 198.1–199.5 °C; IR (KBr) ν_{max} (cm^{-1}): 1640 (C=O), 3580–3685 (OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 3.03 (s, 3H, CH_3), 7.2–7.6 (m, 6H, Ar–H), 11.52 (bs, 1H, OH). MS (EI): *m/z* (85%): M^+ 264.5; Anal. Calcd. for $C_{14}H_{10}ClFO_2$ (264.5): C, 63.53; H, 3.81; Cl, 13.39; F, 7.18. Found: C, 63.41; H, 3.72; Cl, 13.22; F, 7.29%.

*6.1.3. General procedure for ethyl 4-aryloylaryloxyacetates (**5a**–**e**)*

To a solution of compounds **4a**–**e** (0.1156 mol) in dry DMF (175 mL), potassium carbonate (0.3468 mol) and ethyl bromoacetate (0.1273 mol) were added and the reaction mass was heated to 60 °C for 3 h. The reaction mass was diluted with ethyl acetate (200 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (100 mL). The organic layer was washed with water (3 × 30 mL), brine (2 × 40 mL), dried over sodium sulfate and concentrated to yield compounds **5a**–**e**.

*6.1.3.1. Synthesis of ethyl [2-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]acetate (**5a**).* To a solution of compound **4a** (31 g, 0.1156 mol) in dry DMF (175 mL), potassium carbonate (47.83 g, 0.3468 mol) and ethyl bromoacetate (21.11 g, 0.1273 mol) were added and the reaction mass was heated to 60 °C and maintained for 3 h. The reaction mass was diluted with ethyl acetate (200 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (100 mL). The organic layer was washed with water (3 × 30 mL), brine (2 × 40 mL), dried over sodium sulfate and concentrated to yield compound **5a** as brown pasty mass.

Yield: 97%; IR (KBr) ν_{max} (cm^{-1}): 1660 (C=O), 1730 (ester, C=O); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 1.16–1.22 (t, 3H, CH_3), 4.14–4.21 (q, 2H, CH_2), 5.02 (s, 2H, OCH_2), 7.64–7.8 (m, 6H, Ar–H). MS (EI): *m/z* (59%): M^+ 354.5; Anal. Calcd. for $C_{17}H_{13}ClF_2O_4$ (354.5): C, 57.56; H, 3.69; Cl, 9.99; F, 10.71. Found: C, 57.41; H, 3.52; Cl, 9.79; F, 10.88%.

Compounds **5b**–**e** were synthesized analogously starting with **4b**–**e** respectively.

5b: Yield: 92%; IR (KBr) ν_{max} (cm^{-1}): 1650 (C=O), 1740 (ester, C=O); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 1.18–1.22 (t, 3H, CH_3), 4.12–4.19 (q, 2H, CH_2), 5.02 (s, 2H, OCH_2), 7.59–7.75 (m, 6H, Ar–H). MS (EI): *m/z* (58%): M^+ 371; Anal. Calcd. for $C_{17}H_{13}Cl_2FO_4$ (371): C, 55.01; H, 3.53; Cl, 19.10; F, 5.12. Found: C, 55.19; H, 3.41; Cl, 19.18; F, 5.23%.

5c: Yield: 91%; IR (KBr) ν_{max} (cm^{-1}): 1660 (C=O), 1765 (ester, C=O); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 1.18–1.22 (t, 3H, CH_3), 4.21–4.12 (q, 2H, CH_2), 5.0 (s, 2H, OCH_2), 7.38–7.85 (m, 6H, Ar–H). MS (EI): *m/z* (57%): M^+ 415.5; Anal. Calcd. for $C_{17}H_{13}BrClFO_4$ (415.5): C, 49.12; H, 3.15; Br, 19.22; Cl, 8.53; F, 4.57. Found: C, 49.28; H, 3.01; Br, 19.13; Cl, 8.62; F, 4.47%.

5d: Yield: 94%; IR (KBr) ν_{max} (cm^{-1}): 1605 (C=O), 1750 (ester, C=O); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 1.16–1.22 (t, 3H, CH_3), 4.14–4.21 (q, 2H, CH_2), 5.0 (s, 2H, OCH_2), 7.21–7.9 (m, 6H, Ar–H). MS (EI): *m/z* (57%): M^+ 462.5; Anal. Calcd. for $C_{17}H_{13}ClFIO_4$ (462.5): C, 44.13; H, 2.83; Cl, 7.66; F, 4.11; I, 27.43. Found: C, 44.23; H, 2.72; Cl, 7.57; F, 4.23; I, 27.31%.

5e: Yield: 96%; IR (KBr) ν_{max} (cm^{-1}): 1610 (C=O), 17,665 (ester, C=O); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 1.19–1.23 (t, 3H, CH_3), 2.4 (s, 3H, Ar–CH_3), 4.2–4.3 (q, 2H, CH_2), 5.1 (s, 2H, OCH_2), 7.1–7.55 (m, 6H, Ar–H). MS (EI): *m/z* (57%): M^+ 350.5; Anal. Calcd. for $C_{18}H_{16}ClFO_4$ (350.5): C, 61.63; H, 4.60; Cl, 10.11; F, 5.42. Found: C, 61.51; H, 4.52; Cl, 10.19; F, 5.29%.

*6.1.4. General procedure for 4-aryloylaryloxyethanoic acids (**6a**–**e**)*

A mixture of compounds **5a**–**e** (0.0532 mol), 10% aqueous sodium hydroxide solution (100 mL) and THF (100 mL) was stirred at room temperature for 1 h. The reaction mass was acidified with 6 N hydrochloric acid (150 mL) and the aqueous layer was extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with brine (3 × 60 mL), dried over anhydrous sodium sulfate and concentrated to achieve compounds **6a**–**e**.

*6.1.4.1. Synthesis of [4-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy] ethanoic acid (**6a**).* A mixture of compound **5a** (18 g, 0.0532 mol), 10% aqueous sodium hydroxide solution (100 mL) and THF (100 mL) was stirred at room temperature for 1 h. The reaction mass was acidified with 6 N hydrochloric acid (150 mL) and the aqueous layer was extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with brine (3 × 60 mL), dried over anhydrous sodium sulfate and concentrated to achieve compound **6a** as white solid.

Yield: 92%; m.p.: 127.3–128.6 °C; IR (KBr) ν_{max} (cm^{-1}): 1660 (C=O), 1738 (acid C=O), 3470–3575 (acid OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.9 (s, 2H, OCH_2), 7.3–7.87 (m, 6H, Ar–H), 13.1 (s, 1H, COOH). MS (EI): *m/z* (55%): M^+ 326.5; Anal. Calcd. for $C_{15}H_9ClF_2O_4$ (326.5): C, 55.15; H, 2.78; Cl, 10.85; F, 11.63. Found: C, 55.25; H, 2.61; Cl, 10.72; F, 11.49%.

Compounds **6b**–**e** were synthesized analogously starting with **5b**–**e** respectively.

6b: Yield: 90.6%; m.p.: 137.7–139.1 °C; IR (KBr) ν_{max} (cm^{-1}): 1640 (C=O), 1750 (acid C=O), 3480–3590 (acid OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.85 (s, 2H, OCH_2), 7.15–7.77 (m, 6H, Ar–H), 12.9 (s, 1H, COOH). MS (EI): *m/z* (53%): M^+ 343; Anal. Calcd. for $C_{15}H_9Cl_2FO_4$ (343): C, 52.50; H, 2.64; Cl, 20.66; F, 5.54. Found: C, 52.59; H, 2.54; Cl, 20.75; F, 5.39%.

6c: Yield: 92%; m.p.: 153.8–155.3 °C; IR (KBr) ν_{max} (cm^{-1}): 1655 (C=O), 1760 (acid C=O), 3490–3595 (acid OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.9 (s, 2H, OCH_2), 7.25–7.88 (m, 6H, Ar–H), 12.8 (s, 1H, COOH). MS (EI): *m/z* (51%): M^+ 387.5; Anal. Calcd. for $C_{15}H_9BrClFO_4$ (387.5): C, 46.48; H, 2.34; Br, 20.62; Cl, 9.15; F, 4.90. Found: C, 46.33; H, 2.46; Br, 20.52; Cl, 9.33; F, 4.79%.

6d: Yield: 88%; m.p.: 139.7–140.8 °C; IR (KBr) ν_{max} (cm^{-1}): 1615 (C=O), 1770 (acid C=O), 3465–3575 (acid OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.8 (s, 2H, OCH_2), 7.1–7.65 (m, 6H, Ar–H), 11.9 (s, 1H, COOH). MS (EI): *m/z* (54%): M^+ 434.5; Anal. Calcd. for $C_{15}H_9ClFIO_4$ (434.5): C, 41.46; H, 2.09; Cl, 8.16; F, 4.37; I, 29.20. Found: C, 41.33; H, 2.19; Cl, 8.29; F, 4.21; I, 29.41%.

6e: Yield: 89%; m.p.: 119.2–120.5 °C; IR (KBr) ν_{max} (cm^{-1}): 1630 (C=O), 1765 (acid C=O), 3415–3545 (acid OH); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 2.4 (s, 3H, CH_3), 4.96 (s, 2H, OCH_2), 7.16–7.77 (m, 6H, Ar–H), 12.65 (s, 1H, COOH). MS (EI): *m/z* (53%): M^+ 322.5; Anal. Calcd. for $C_{16}H_{12}ClFO_4$ (322.5): C, 59.55; H, 3.75; Cl, 10.99; F, 5.89. Found: C, 59.63; H, 3.61; Cl, 10.78; F, 5.72%.

*6.1.5. General procedure for 4-aryloylaryloxyacethydrazides (**7a**–**e**)*

Hydrazine hydrate (0.3372 mol) was added to a solution of compounds **6a**–**e** (0.0562 mol) in ethanol (100 mL) at 0 °C and stirred the reaction mixture at the same temperature for 1 h. A white solid was separated out, which was quenched with water (100 mL), filtered and washed with water (50 mL). Finally, solid was dried under vacuum to obtain compounds **7a**–**e**.

*6.1.6. Synthesis of 2-[4-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]acethydrazide (**7a**)*

Hydrazine hydrate (16.90 g, 0.3372 mol) was added to a solution of compound **6a** (19 g, 0.0562 mol) in ethanol (100 mL) at 0 °C and stirred the reaction mixture at the same temperature for 1 h. A white solid was separated out, which was quenched with

water (100 mL), filtered and washed with water (50 mL). Finally, solid was dried under vacuum to obtain compound **7a** as white needle.

Yield: 79%; m.p.: 107.5–109.1 °C; IR (KBr) ν_{max} (cm^{-1}): 1610 (C=O), 1645 (amide, C=O), 3100–3205 (NH–NH_2); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.35 (bs, 2H, NH_2), 4.69 (s, 2H, OCH_2), 7.2–7.86 (m, 6H, Ar–H), 9.32 (bs, 1H, CONH). MS (EI): *m/z* (42%): M^+ 340.5; Anal. Calcd. for $C_{15}H_{11}ClF_2N_2O_3$ (340.5): C, 52.88; H, 3.25; Cl, 10.41; F, 11.15; N, 8.22. Found: C, 52.75; H, 3.38; Cl, 10.29; F, 11.24; N, 8.11%.

Compounds **7b**–**e** were synthesized analogously starting with **6b**–**e** respectively.

7b: Yield: 75.66%; m.p.: 141.4–142.9 °C; IR (KBr) ν_{max} (cm^{-1}): 1625 (C=O), 1655 (amide, C=O), 3150–3255 (NH–NH_2); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.25 (bs, 2H, NH_2), 4.70 (s, 2H, OCH_2), 7.1–7.75 (m, 6H, Ar–H), 9.30 (bs, 1H, CONH). MS (EI): *m/z* (43%): M^+ 357; Anal. Calcd. for $C_{15}H_{11}Cl_2FN_2O_3$ (357): C, 50.44; H, 3.10; Cl, 19.85; F, 5.32; N, 7.84. Found: C, 50.31; H, 3.22; Cl, 19.71; F, 5.21; N, 7.72%.

7c: Yield: 66%; m.p.: 137.2–138.6 °C; IR (KBr) ν_{max} (cm^{-1}): 1635 (C=O), 1660 (amide, C=O), 3155–3270 (NH–NH_2); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.35 (bs, 2H, NH_2), 4.70 (s, 2H, OCH_2), 7.2–7.65 (m, 6H, Ar–H), 9.32 (bs, 1H, CONH). MS (EI): *m/z* (41%): M^+ 401.5; Anal. Calcd. for $C_{15}H_{11}BrClFN_2O_3$ (401.5): C, 44.86; H, 2.76; Br, 19.90; Cl, 8.83; F, 4.73; N, 6.98. Found: C, 44.74; H, 2.62; Br, 19.78; Cl, 8.71; F, 4.61; N, 6.85%.

7d: Yield: 78%; m.p.: 120.1–123.2 °C; IR (KBr) ν_{max} (cm^{-1}): 1615 (C=O), 1615 (amide, C=O), 3120–3250 (NH–NH_2); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.38 (bs, 2H, NH_2), 4.72 (s, 2H, OCH_2), 7.15–7.55 (m, 6H, Ar–H), 9.32 (bs, 1H, CONH). MS (EI): *m/z* (40%): M^+ 448.5; Anal. Calcd. for $C_{15}H_{11}ClFIN_2O_3$ (448.5): C, 40.16; H, 2.47; Cl, 7.90; F, 4.23; I, 28.29; N, 6.24. Found: C, 40.03; H, 2.33; Cl, 7.79; F, 4.15; I, 28.18; N, 6.14%.

7e: Yield: 80%; m.p.: 78.3–79.7 °C; IR (KBr) ν_{max} (cm^{-1}): 1605 (C=O), 1610 (amide, C=O), 3135–3270 (NH–NH_2); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 2.40 (s, 2H, CH_3), 4.37 (bs, 2H, NH_2), 4.69 (s, 2H, OCH_2), 7.05–7.69 (m, 6H, Ar–H), 9.31 (bs, 1H, CONH). MS (EI): *m/z* (43%): M^+ 336.5; Anal. Calcd. for $C_{16}H_{14}ClFN_2O_3$ (336.5): C, 57.07; H, 4.19; Cl, 10.53; F, 5.64; N, 8.32. Found: C, 57.17; H, 4.11; Cl, 10.41; F, 5.78; N, 8.47%.

*6.1.7. General procedure for N,N-di(2-(4-aryloylaryloxy)acetyl) hydrazines (**8a**–**j**)*

To a solution of compounds **6a**–**e** (0.0032 mol) in DCM (20 mL), 2,6-dimethylpyridine (0.0107 mol) and TBTU (0.00323 mol) were added at room temperature. Finally, compounds **7a**–**e** (0.00294 mol) were added to the reaction mixture and stirred at room temperature for 12 h. The reaction mixture was quenched with 10% sodium bicarbonate solution (20 mL) and stirred for 30 min. The solid precipitate was filtered, washed with water (20 mL) and dried to yield compounds **8a**–**j**.

*6.1.7.1. Synthesis of N,N-di[di(2-chloro-6-fluoro-4-(4-fluoro-benzoyl) phenoxy)]acetyl hydrazide (**8a**).* To a solution of compound **6a** (1.05 g, 0.0032 mol) in DCM (20 mL), 2,6-dimethylpyridine (1 g, 0.0107 mol) and TBTU (1.04 g, 0.00323 mol) were added at room temperature. Finally, compound **7a** (1 g, 0.00294 mol) was added to the reaction mixture and stirred at room temperature for 12 h. The reaction mixture was quenched with 10% sodium bicarbonate solution (20 mL) and stirred for 30 min. The solid precipitate was filtered, washed with water (20 mL) and dried to yield compound **8a** as white solid.

Yield: 81%; m.p.: 194.8–196.2 °C; IR (KBr) ν_{max} (cm^{-1}): 1690 (C=O), 1610 (amide, C=O), 3700–3500 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.90 (s, 4H, $2CH_2$), 7.1–7.87 (m, 12H, Ar–H), 10.36 (bs, 2H, 2NH). MS (EI): *m/z* (61%): M^+ 649; Anal. Calcd. for $C_{30}H_{18}Cl_2F_4N_2O_6$ (649): C, 55.49; H, 2.79; Cl, 10.92; F, 11.70; N, 4.31. Found: C, 55.37; H, 2.88; Cl, 10.83; F, 11.77; N, 4.43%.

Similarly compounds **8b**–**j** were synthesized starting from compounds **6b**–**e** and **7a**–**e**.

8b: Yield: 78%; m.p.: 189.2–190.5 °C; IR (KBr) ν_{max} (cm^{-1}): 1680 (C=O), 1605 (amide, C=O), 3710–3510 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.85 (s, 4H, $2CH_2$), 7.15–7.85 (m, 12H, Ar–H), 10.38 (bs, 2H, 2NH). MS (EI): *m/z* (60%): M^+ 665.5; Anal. Calcd. for $C_{30}H_{18}Cl_3F_3N_2O_6$ (665.5): C, 54.12; H, 2.72; Cl, 15.97; F, 8.56; N, 4.21. Found: C, 54. 21; H, 2.63; Cl, 15.84; F, 8.42; N, 4.13%.

8c: Yield: 79%; m.p.: 209.1–210.4 °C; IR (KBr) ν_{max} (cm^{-1}): 1685 (C=O), 1610 (amide, C=O), 3720–3520 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.86 (s, 4H, $2CH_2$), 7.0–7.79 (m, 12H, Ar–H), 10.4 (bs, 2H, 2NH). MS (EI): *m/z* (61%): M^+ 710; Anal. Calcd. for $C_{30}H_{18}BrCl_2F_3N_2O_6$ (710): C, 50.73; H, 2.55; Br, 11.25; Cl, 9.98; F, 8.02; N, 3.94. Found: C, 50.61; H, 2.47; Br, 11.13; Cl, 9.86; F, 8.16; N, 3.82%.

8d: Yield: 78%; m.p.: 189.3–190.7 °C; IR (KBr) ν_{max} (cm^{-1}): 1675 (C=O), 1615 (amide, C=O), 3725–3525 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.85 (s, 4H, $2CH_2$), 7.1–7.75 (m, 12H, Ar–H), 10.8 (bs, 2H, 2NH). MS (EI): *m/z* (59%): M^+ 757; Anal. Calcd. for $C_{30}H_{18}Cl_2F_3N_2O_6$ (757): C, 47.58; H, 2.40; Cl, 9.36; F, 7.53; I, 16.76; N, 3.70. Found: C, 47.46; H, 2.49; Cl, 9.25; F, 7.61; I, 16.66; N, 3.79%.

8e: Yield: 84%; m.p.: 178.5–180.1 °C; IR (KBr) ν_{max} (cm^{-1}): 1690 (C=O), 1620 (amide, C=O), 3735–3530 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 2.4 (s, 3H, CH_3), 4.8 (s, 4H, $2CH_2$), 7.2–7.72 (m, 12H, Ar–H), 10.36 (bs, 2H, 2NH). MS (EI): *m/z* (58%): M^+ 645; Anal. Calcd. for $C_{31}H_{21}Cl_2F_3N_2O_6$ (645): C, 57.69; H, 3.28; Cl, 10.99; F, 8.83; N, 4.34. Found: C, 57.58; H, 3.36; Cl, 10.88; F, 8.74; N, 4.25%.

8f: Yield: 79%; m.p.: 193.2–194.4 °C; IR (KBr) ν_{max} (cm^{-1}): 1695 (C=O), 1605 (amide, C=O), 3720–3520 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.88 (s, 4H, $2CH_2$), 7.12–7.73 (m, 12H, Ar–H), 10.38 (bs, 2H, 2NH). MS (EI): *m/z* (59%): M^+ 682; Anal. Calcd. for $C_{30}H_{18}Cl_4F_2N_2O_6$ (682): C, 52.81; H, 2.66; Cl, 20.78; F, 5.57; N, 4.11. Found: C, 52.72; H, 2.55; Cl, 20.69; F, 5.66; N, 4.21%.

8g: Yield: 84%; m.p.: 178.3–179.8 °C; IR (KBr) ν_{max} (cm^{-1}): 1680 (C=O), 1615 (amide, C=O), 3735–3525 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 2.3 (s, 3H, CH_3), 4.86 (s, 4H, $2CH_2$), 7.05–7.75 (m, 12H, Ar–H), 10.35 (bs, 2H, 2NH). MS (EI): *m/z* (61%): M^+ 661.5; Anal. Calcd. for $C_{31}H_{21}Cl_3F_2N_2O_6$ (661.5): C, 56.26; H, 3.20; Cl, 16.07; F, 5.74; N, 4.23. Found: C, 56.35; H, 3.28; Cl, 16.15; F, 5.65; N, 4.31%.

8h: Yield: 77%; m.p.: 180.3–181.6 °C; IR (KBr) ν_{max} (cm^{-1}): 1685 (C=O), 1610 (amide, C=O), 3745–3530 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.75 (s, 4H, $2CH_2$), 7.05–7.8 (m, 12H, Ar–H), 10.6 (bs, 2H, 2NH). MS (EI): *m/z* (60%): M^+ 773.5; Anal. Calcd. for $C_{30}H_{18}Cl_3F_2IN_2O_6$ (773.5): C, 46.57; H, 2.34; Cl, 13.75; F, 4.91; I, 16.40; N, 3.62. Found: C, 46.64; H, 2.26; Cl, 13.83; F, 4.84; I, 16.47; N, 3.55%.

8i: Yield: 80%; m.p.: 182.4–183.8 °C; IR (KBr) ν_{max} (cm^{-1}): 1670 (C=O), 1600 (amide, C=O), 3715–3520 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.85 (s, 4H, $2CH_2$), 7.0–7.79 (m, 12H, Ar–H), 11.0 (bs, 2H, 2NH). MS (EI): *m/z* (62%): M^+ 726.5; Anal. Calcd. for $C_{30}H_{18}BrCl_3F_2N_2O_6$ (726.5): C, 49.58; H, 2.50; Br, 10.99; Cl, 14.64; F, 5.23; N, 3.85. Found: C, 49.49; H, 2.56; Br, 10.89; Cl, 14.77; F, 5.31; N, 3.78%.

8j: Yield: 82%; m.p.: 201.7–203.1 °C; IR (KBr) ν_{max} (cm^{-1}): 1685 (C=O), 1605 (amide, C=O), 3720–3535 (NH–NH); ^{1}H NMR (400 MHz) (DMSO-d_6) δ (ppm): 4.88 (s, 4H, $2CH_2$), 6.9–7.71 (m, 12H, Ar–H), 10.35 (bs, 2H, 2NH). MS (EI): *m/z* (61%): M^+ 771; Anal. Calcd. for $C_{30}H_{18}Br_2Cl_2F_2N_2O_6$ (771): C, 46.72; H, 2.35; Br, 20.72; Cl, 9.19; F, 4.93; N, 3.63. Found: C, 46.64; H, 2.42; Br, 20.65; Cl, 9.28; F, 4.82; N, 3.71%.

*6.1.8. General procedure for 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles (**9a–j**)*

To a solution of compounds **8a–j** (0.0023 mol) in DCM (20 mL), pyridine (0.0069 mol) and triflic anhydride (0.0051 mol) were added at 0 °C and the reaction mixture was stirred at 0 °C for 3 h. The reaction mass was diluted with DCM (20 mL), the organic layer was washed with 10% sodium bicarbonate (3 × 10 mL), water (3 × 10 mL) and brine (3 × 10 mL). Finally, the organic layer was dried over sodium sulfate and concentrated to yield compounds **9a–j**.

*6.1.8.1. Synthesis of 2,5-di[2-fluoro-4-(4-fluoro)benzoyl-6-chlorophenoxymethyl]1,3,4-oxadiazole (**9a**).* To a solution of compound **8a** (1.5 g, 0.0023 mol) in DCM (20 mL), pyridine (0.56 g, 0.0069 mol) and triflic anhydride (1.44 g, 0.0051 mol) were added at 0 °C and the reaction mixture was stirred at 0 °C for 3 h. After the completion of the reaction monitored by TLC, the reaction mass was diluted with DCM (20 mL), the organic layer was washed with 10% sodium bicarbonate (3 × 10 mL), water (3 × 10 mL) and brine (3 × 10 mL). Finally, the organic layer was dried over sodium sulfate and concentrated to yield a brown gummy mass. The crude product was purified by column chromatography using silica gel as stationary phase and hexane:ethyl acetate as mobile phase to achieve compound **9a** as white solid.

Yield; 73%; m.p.: 129.5–130.2 °C; IR (Nujol) ν_{max} (cm^{-1}): 1153 (C–O–C linkage), 1658 (C=O), 1683 cm^{-1} (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.6 (s, 4H, $2CH_2$), 7.05–7.81 (m, 12H, Ar–H). MS (EI): *m/z* (70%): M^+ 631; Anal. Calcd. for $C_{30}H_{16}Cl_2F_4N_2O_5$ (631): C, 57.07; H, 2.55; Cl, 11.23; F, 12.04; N, 4.44. Found: C, 57.17; H, 2.47; Cl, 11.31; F, 12.13; N, 4.36%.

Similarly compounds **9b–j** were synthesized starting from compounds **8b–j**.

9b: Yield; 76%: m.p.: 88.9–90.2 °C; IR (Nujol) ν_{max} (cm^{-1}): 1158 (C–O–C linkage), 1660 (C=O), 1685 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.6 (s, 4H, $2CH_2$), 7.1–7.79 (m, 12H, Ar–H). MS (EI): *m/z* (71%): M^+ 647.5; Anal. Calcd. for $C_{30}H_{16}Cl_3F_3N_2O_5$ (647.5): C, 55.62; H, 2.49; Cl, 16.42; F, 8.80; N, 4.32. Found: C, 55.71; H, 2.41; Cl, 16.51; F, 8.72; N, 4.23%.

9c: Yield: 71%; m.p.: 88.9–90.2 °C; IR (Nujol) ν_{max} (cm^{-1}): 1155 (C–O–C linkage), 1665 (C=O), 1680 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.55 (s, 4H, $2CH_2$), 7.05–7.78 (m, 12H, Ar–H). MS (EI): *m/z* (69%): M^+ 692; Anal. Calcd. for $C_{30}H_{16}$ $BrCl_2F_3N_2O_5$ (692): C, 52.05; H, 2.33; Br, 11.54; Cl, 10.24; F, 8.23; N, 4.05. Found: C, 52.15; H, 2.26; Br, 11.46; Cl, 10.17; F, 8.31; N, 4.11%.

9d: Yield: 75.2%; m.p.: 88.9–90.6 °C; IR (Nujol) ν_{max} (cm^{-1}): 1160 (C–O–C linkage), 1650 (C=O), 1685 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.6 (s, 4H, $2CH_2$), 7.1–7.75 (m, 12H, Ar–H). MS (EI): *m/z* (68%): M^+ 739; Anal. Calcd. for $C_{30}H_{16}Cl_2F_3IN_2O_5$ (739): C, 48.74; H, 2.18; Cl, 9.59; F, 7.71; I, 17.17; N, 3.79. Found: C, 48.66; H, 2.24; Cl, 9.51; F, 7.63; I, 17.27; N, 3.69%.

9e: Yield: 68.5%; m.p.: 72.0–73.4 °C; IR (Nujol) ν_{max} (cm^{-1}): 1150 (C–O–C linkage), 1635 (C=O), 1690 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 2.40 (s, 3H, CH_3), 5.59 (s, 4H, $2CH_2$), 7.05–7.8 (m, 12H, Ar–H). MS (EI): *m/z* (69%): M^+ 627; Anal. Calcd. for $C_{31}H_{19}Cl_2F_3N_2O_5$ (627): C, 59.35; H, 3.05; Cl, 11.30; F, 9.08; N, 4.47. Found: C, 59.43; H, 3.14; Cl, 11.21; F, 9.17; N, 4.55%.

9f: Yield: 75.3%; m.p.: 110.4–111.7 °C; IR (Nujol) ν_{max} (cm^{-1}): 1152 (C–O–C linkage), 1627 (C=O), 1685 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.6 (s, 4H, $2CH_2$), 7.1–7.77 (m, 12H, Ar–H). MS (EI): *m/z* (66%): M^+ 664; Anal. Calcd. for $C_{30}H_{16}Cl_4F_2N_2O_5$ (664): C, 54.24; H, 2.43; Cl, 21.35; F, 5.72; N, 4.22. Found: C, 54.31; H, 2.35; Cl, 21.26; F, 5.61; N, 4.32%.

9g: Yield: 72%; m.p.: 87.6–89.9 °C; IR (Nujol) ν_{max} (cm^{-1}): 1100 (C–O–C linkage), 1600 (C=O), 1675 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 2.3 (s, 3H, CH_3), 5.6 (s, 4H, $2CH_2$), 7.05–7.79 (m, 12H, Ar–H). MS (EI): *m/z* (67%): M^+ 643.5; Anal. Calcd. for $C_{31}H_{19}Cl_3F_2N_2O_5$ (643.5): C, 57.83; H, 2.97; Cl, 16.52; F, 5.90; N, 4.35. Found: C, 57.74; H, 2.88; Cl, 16.41; F, 5.81; N, 4.26%.

9h: Yield: 71%; m.p.: 118.3–119.6 °C; IR (Nujol) ν_{max} (cm^{-1}): 1105 (C–O–C linkage), 1610 (C=O), 1690 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.55 (s, 4H, $2CH_2$), 7.12–7.72 (m, 12H, Ar–H). MS (EI): *m/z* (69%): M^+ 755.5; Anal. Calcd. for $C_{30}H_{16}Cl_3F_2IN_2O_5$ (755.5): C, 47.68; H, 2.13; Cl, 14.07; F, 5.03; I, 16.79; N, 3.71. Found: C, 47.61; H, 2.22; Cl, 14.15; F, 5.14; I, 16.68; N, 3.63%.

9i: Yield: 73%; m.p.: 130.6–131.8 °C; IR (Nujol) ν_{max} (cm^{-1}): 1110 (C–O–C linkage), 1615 (C=O), 1685 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.6 (s, 4H, $2CH_2$), 7.12–7.79 (m, 12H, Ar–H). MS (EI): *m/z* (67%): M^+ 708.5; Anal. Calcd. for $C_{30}H_{16}BrCl_3F_2N_2O_5$ (708.5): C, 50.84; H, 2.28; Br, 11.27; Cl, 15.01; F, 5.36; N, 3.95. Found: C, 50.77; H, 2.35; Br, 11.37; Cl, 15.11; F, 5.27; N, 3.88%.

9j: Yield: 71%; m.p.: 132.3–133.6 °C; IR (Nujol) ν_{max} (cm^{-1}): 1106 (C–O–C linkage), 1610 (C=O), 1675 (C=N); ^1H NMR (400 MHz) (DMSO-d_6) δ (ppm): 5.57 (s, 4H, $2CH_2$), 7.05–7.78 (m, 12H, Ar–H). MS (EI): *m/z* (68%): M^+ 753; Anal. Calcd. for $C_{30}H_{16}Br_2Cl_2F_2N_2O_5$ (753): C, 47.84; H, 2.14; Br, 21.22; Cl, 9.41; F, 5.04; N, 3.72. Found: C, 47.75; H, 2.22; Br, 21.31; Cl, 9.48; F, 5.12; N, 3.65%.

6.2. Biological study

The human chronic myelogenous leukemia (CML) K562 and CEM were selected for the purpose of preliminary anti-cancer screening of newly synthesized compounds. To assess the cytotoxicity, trypan blue dye exclusion assay, MTT assay and LDH assay were employed. A DNA fragmentation assay, which is an indicator of apoptosis, was also performed.

6.2.1. Cell lines and culture

Human cell lines, K562 and CEM (T-cell leukemia) were purchased from National Center for Cell Science, Pune, India. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of Penicillin, and 100 μg of streptomycin/mL and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

6.2.2. In vitro cell viability and cell proliferation assay: trypan blue exclusion assay

Cell viability was monitored by the trypan blue exclusion assay as reported earlier [20]. To determine the effect of compounds **9a–j** on viability of K562 or CEM cells, approximately 0.75×10^5 cells/mL were seeded in a 6-well tissue culture plate for 24 h and compounds **9a–j** were added at a concentration of 10, 50, 100 and 250 μM. 5-Fluorouracil treated cells were used as positive control. Cells were collected at intervals of 24 h and resuspended in 0.4% trypan blue (viable-unstained and non viable-blue). The number of viable cells were counted using hemocytometer chamber.

6.2.3. MTT assay

Cell proliferation was further assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the ability of viable cells to metabolize a yellow tetrazolium salt to violet formazan. Exponentially growing K562, or CEM cells (1×10^4 cells/well) were plated in triplicates and incubated with 10, 50, 100 and 250 μM of compounds **9a–j**. Cells were harvested after 48 and 72 h of treatment and incubated with MTT (0.5 mg/mL) at 37 °C. The blue MTT formazan precipitate was then solubilized in detergent (50% final concentration of *N*,*N*-dimethylformamide and 10% of sodium dodecyl sulfate). Absorbance was measured at 570 nm using ELISA plate reader. The mean absorbance of culture medium was used as the blank and was subtracted. IC_{50} values (concentration of compound causing 50% inhibition of cell growth) were estimated after 72 h of title compounds treatment (Table 1).

6.2.4. LDH release assay

Lactate dehydrogenase (LDH) release is an indicator of membrane integrity and hence cell injury. LDH assay was performed as per standard protocols [21] to assess the LDH release in the culture media following the treatment with compounds **9b**, **9e**, **9i** and **9j** (10, 50 and 100 μM) on K562 cells for 24 and 48 h. The intracellular LDH was determined after lysing the cells by freezing at −80 °C and rapid thawing. The LDH release was measured at an absorbance of 490 nm. The percentage of LDH release was calculated as: (LDH activity in media)/(LDH activity in media + LDH activity in total cells) × 100%. The LDH release was plotted as graph as shown in Fig. 1.

6.2.5. DNA fragmentation assay

DNA fragmentation was performed for elucidating the mode of action of the investigated compounds, especially with respect to induction of oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis. During the apoptotic process, activated nucleases degrade the higher order chromatin structure of DNA into mono- and oligonucleosomal DNA-fragments. Apoptotic degradation of DNA was analyzed by agarose gel electrophoresis [20]. Briefly, K562 cells were cultured in presence of **9b** or **9i** at 10, 50 and 100 μM for 72 h. Cells were harvested and genomic DNA was extracted using standard protocol. DNA was resuspended in 250 μl of TE buffer. The DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining and photographed.

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