Synthesis and Cytotoxicity Evaluation of *N*-(5-(Substituted-benzylthio)-1,3,4-thiadiazole-2-yl) -2-*p*-nitrophenylacetamide Derivatives as Potential Anticancer Agents

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ABSTRACT: *Cancer is a big global problem and is one of the top and main causes of mortality in developed countries. Many of the current treatments and anticancer therapeutics have problems with severe side effects and on the other hand, the drug resistance is also another obstacle in the cancer chemotherapy. Hence, there is a strong demand for the discovery and development of effective new antineoplastic therapies. According to the in vitro effectiveness of 1,3,4-thiadiazole based compounds as anticancer agents, new 1,3,4-thiadiazole based derivatives with various electron withdrawing and electron donating moieties were synthesized and tested by MTT assay against three cancerous cell lines. PC3 (Prostate cancer), U87-C-531 (Glioblastoma) and MDA-MB-231 (Breast cancer) cell lines were applied for MTT assay and obtained results were compared to imatinib. Study of the structure activity relationship of prepared compounds showed electron withdrawing substituents such as Cl, F and NO_2 enhanced the anticancer properties compared to compound without any substituent (compound 3l) or compounds with electron donating (methoxy) substituent (compounds 3j and 3k). Totally, compound 3a (IC_{50} = 10.6 µM) showed superior activity against PC3 cell line and compounds 3d (IC_{50} = 10.3 µM), 3h (IC_{50} = 12.5 µM) and 3j (IC_{50} = 11.3 µM) exhibited higher activity against MDA-MB-231 cell line compared to imatinib as reference drug.*

KEYWORDS: *Synthesis; 1,3,4-Thiadiazole; MTT; Anticancer.*

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INTRODUCTION

Cancer is a collection of several disorders afflicting all parts of the body. Even a single type of cancer presents itself differently in different individuals. The disease is the result of the main failure in the biochemical signaling networks that drive the normal cell. Accelerated growth and reduced death are two characteristic features of all neoplastic cells [1, 2]. For decades, conventional chemotherapy has been the most common type of anticancer pharmacotherapy [3]. Cancer chemotherapy has been one of the major advances in area of medicine in the last few decades. However, the drugs administered for chemotherapy have a narrow therapeutic index and therefore high incidence of unwanted side effects [4].

1,3,4-Thiadiazole is a five-membered ring system that exerts a wide variety of biological activities. 1,3,4-Thiadiazole displays a broad spectrum of biological activity. The lower toxicity and *in vivo* stability of 1,3,4-thiadiazole nucleus is attributed to its aromaticity. 1,3,4-Thiadiazole has exhibited potential antiglaucoma, antiinflammatory, antitumor, antiulcer, antibacterial, antiviral, analgesic, antiepileptic, antifungal and radioprotective activities. The marketed drugs like acetazolamide (diuretic), sulfaethidole (antibacterial), cefazolin (antibacterial), etc. have 1,3,4-thiadiazole ring [5-11].

Numerous chemical structures with 1,3,4-Thiadiazole ring have been reported with potential anticancer activity (Fig. 1) [12-19]. According to the report of *Maurizio Botta et. al.* as well as the continuation of our previous investigations towards the discovery of new derivatives of 1,3,4-thiadiazole ring as potent dual inhibitors of abl and src tyrosine kinases with anticancer property (Fig. 2), we encouraged to synthesize new analogs of these series [20, 21]. Subsequently, the evaluation of their preliminary anticancer activity was carried out *in vitro* against three cancer cell lines.

EXPERIMENTAL SECTION

Chemistry

All starting materials, reagents, and solvents were purchased from commercial vendors such as Merck and Sigma-Aldrich companies. The purity of the prepared compounds was confirmed by Thin Layer Chromatography (TLC) using various solvents of different polarities. Merck silica gel 60 F_{254} plates were applied for analytical TLC. Column chromatography was performed on Merck silica gel (70-230 mesh) for purification of intermediate and final compounds. ^{1}H-NMR spectra were recorded using a Varian 400 spectrometer, and chemical shifts are expressed as δ (ppm) with tetramethylsilane (TMS) as internal standard. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks). Melting points were determined on a Kofler hot stage apparatus and are uncorrected. The mass spectra were run on a Finigan TSQ-70 spectrometer (Finigan, USA) at 70 eV. All intended compounds 3a-3l were prepared according to Scheme 1.

Synthesis of N-(5-Mercapto-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (2)

In a flat bottom flask (250 mL), *p*-nitrophenylacetic acid (MW: 181.15 g/mol, 2.7 g, 15 mmol), *N*-Hydroxybenzotriazole (HOBt) (MW: 135.15 g/mol, 2 g, 15 mmol) and EDC hydrochloride (MW: 191.70 g/mol, 2.88 g, 15 mmol) were stirred in acetonitrile (30 mL) for 30-45 minutes. Then, 5-amino-1,3,4-thiadiazole-2-thiol (MW: 133.19 g/mol, 2 g, 15 mmol) was added and the stirring was continued for 24 hours. Acetonitrile was evaporated and equal portions of ethylacetate and water (30 mL) was added. The aqueous phase was removed and the ethylacetate phase was washed by sodium bicarbonate, sulfuric acid, and brine. The separated organic phase was dried using anhydrous sodium sulfate. After removing the sodium sulfate salt by filtration, ethylacetate was evaporated under reduced pressure and a yellowish powder was obtained and washed by diethyl ether [20-23].

Yield: 34%, mp: 238 °C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.32 (s, 1H, -SH), 3.95 (s, 2H, -CH_2CO-), 7.58 (d, 2H, *J* = 8Hz), 8.19 (d, 2H, *J* = 8Hz, 4-nitrophenyl), 13.2 (brs, 1H, NH). MS (*m/z*, %): M^++1: 297 (50), 296 (60), 136 (40), 133 (100), 90 (50), 89 (75), 78 (60), 63 (25).

General procedure for synthesis of compounds 3a-3l

N-(5-Mercapto-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl) acetamide (2) (MW: 296.32 g/mol, 0.2 g, 0.67 mmol) of and KOH (MW: 56 g/mol, 0.038 g, 0.67 mmol) KOH were stirred and heated for 5 minutes in absolute ethanol (20 mL) as solvent then, the equivalent amount of appropriate benzyl chloride derivative was added. The reaction was refluxed for 24 hours and the progress was checked by thin layer chromatography and excess

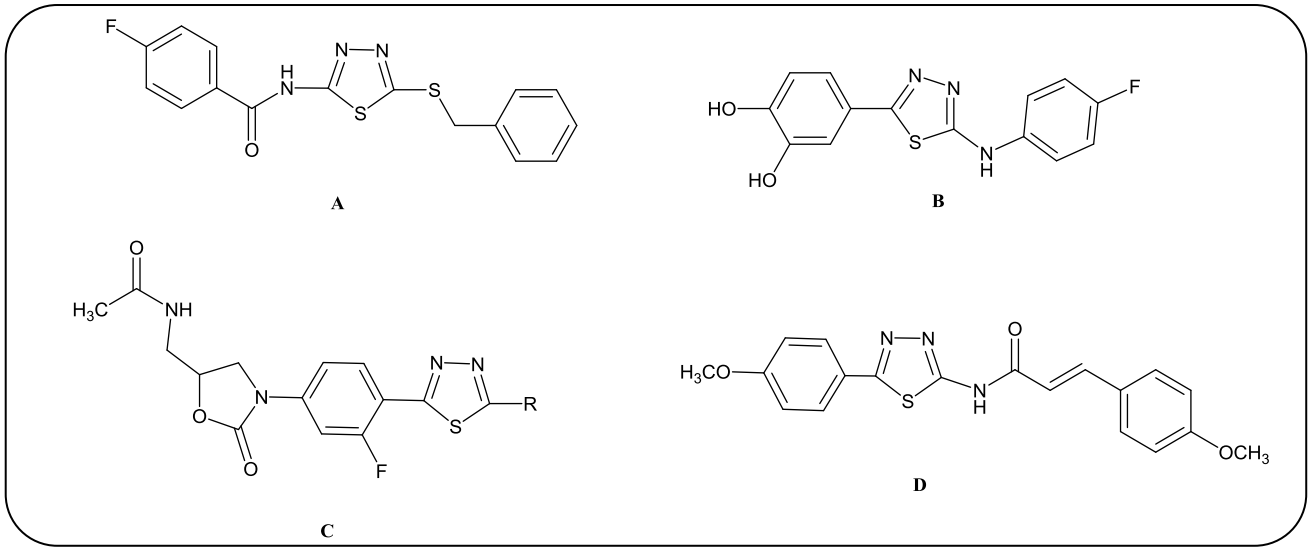


Fig. 1: Structures of some 1,3,4-thiadiazole compounds with anticancer activity.

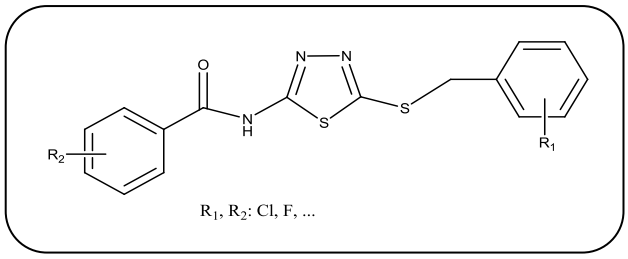


Fig. 2: Total Structure of 5-amino-1,3,4-thiadiazole-2-thiol derivatives as anticancer agents.

amount of benzyl chloride derivative was added in some cases. The reaction was cooled by crushed ice and the creamy precipitate was filtered.

N-(5-(2-Chlorobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3a)

Yield: 34%, mp: 248 °C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.96 (s, 2H, -CH_2CO-), 4.54 (s, 2H, -CH_2S-), 7.24(m, 2-Chlorophenyl), 7.45(m, 2-Chlorophenyl), 7.59(d, 2H, *J* = 8 Hz, 4-Nitrophenyl), 8.18(d, 2H, *J* = 8 Hz, 4-Nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3221, 2851, 2739, 1697, 1575, 1515, 1467, 1344, 1302, 1188, 1047, 959, 827, 757, 727.

N-(5-(3-Chlorobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3b)

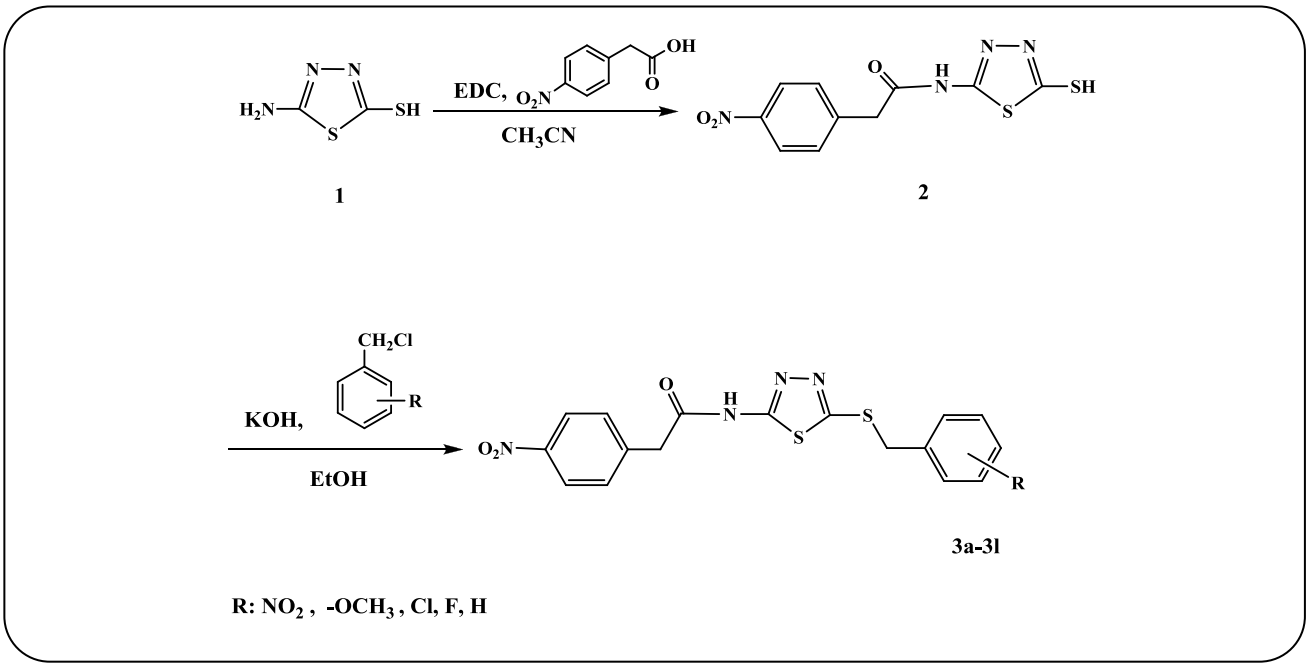
Yield: 43%, mp: 256 °C, ^{1}H NMR (DMSO-d_6 , 400 MHz) δ: 4.00 (s, 2H, -CH_2CO-), 4.47 (s, 2H, -CH_2S-), 7.33 (m, 3-chlorophenyl), 7.46 (s, 1H, H_2-3-chlorophenyl), 7.59 (d, 2H, *J* = 8 Hz, 4-nitrophenyl), 8.19 (d, 2H, *J* = 8 Hz, 4-nitrophenyl), 12.95 (brs, NH). IR (KBr, cm^{-1}) ῡ: 3417, 2918, 2849, 1693, 1566, 1514, 1434, 1350, 1221, 1088, 961, 831, 719.

N-(5-(4-Chlorobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3c)

Yield: 30.7%, mp: 257 °C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.99 (s, 2H, -CH_2CO-), 4.46 (s, 2H, -CH_2S-), 7.35 (d, 2H, *J* = 8 Hz, 4-chlorophenyl), 7.41 (d, 2H, *J* = 8 Hz, 4-chlorophenyl), 7.59 (d, 2H, *J* = 8 Hz, 4-nitrophenyl), 8.19 (d, 2H, *J* = 8 Hz, 4-nitrophenyl), 12.9 (brs, NH). IR (KBr, cm^{-1}) ῡ: 3253, 3165, 3041, 2956, 2854, 1695, 1568, 1508, 1344, 1300, 1230, 1170, 1060, 1016, 810, 755. MS (*m/z*, %): M^++2: 422 (68), M^++1: 421 (70), M^+: 420 (60), 388 (20), 284 (25), 182 (40), 125 (100), 89 (28).

N-(5-(2-Fluorobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3d)

Yield: 37%, mp: 193 °C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.13 (s, 2H, -CH_2CO-), 4.46 (s, 2H, -CH_2-S-), 7.17 (d, *J* = 8 Hz, 2-Fluorophenyl), 7.61 (d, *J* = 8 Hz, 4-Nitrophenyl), 7.73 (d, *J* = 12 Hz, 2-Fluorophenyl), 7.80 (d, *J* = 8 Hz, 2-Fluorophenyl), 7.87 (d, *J* = 12 Hz, 2-Fluorophenyl), 8.17 (d, *J* = 8 Hz, 4-Nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3309, 3253, 3041, 2924, 2906, 2852, 1693, 1620, 1514, 1480, 1344, 1300, 1230, 1170, 1080, 750. MS (*m/z*, %): M^++1: 405 (55), M^+: 404 (60), 388 (20), 166 (60), 136 (55), 121 (30), 109 (100), 89 (60), 78 (40), 63 (20).



Scheme 1: Synthetic procedure for preparation of compounds 3a-3l.

N-(5-(3-Fluorobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3e)

Yield: 35%, mp: 248 ºC, ^{1}H NMR (DMSO-d_6 , 400 MHz) δ: 3.94 (s, 2H, -CH_2CO-), 4.43 (s, 2H, -CH_2S-), 6.96 (t, 2-Flourophenyl), 7.09 (d, *J* = 8 Hz, 4-Flourophenyl), 7.15 (d, *J* = 8 Hz, 4-Flourophenyl), 7.27 (t, 2-Flourophenyl), 7.56 (d, 2H, *J* = 8 Hz, 4-Nitrophenyl), 8.18 (d, 2H, *J* = 8 Hz, 4-Nitrophenyl), 12.9 (brs, NH). IR (KBr, cm^{-1}) ῡ: 3159, 2848, 2726, 1693, 1566, 1515, 1488, 1347, 1305, 1261, 1176, 831, 721.

N-(5-(4-Fluorobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3f)

Yield: 74%, mp: 239 ºC, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.07 (s, 2H, -CH_2CO-), 4.41 (s, 2H, -CH_2-S-), 6.96 (m, 2H, $H_{2,6}$-4-Fluorobenzyl), 7.33 (m, 2H, $H_{3,5}$-4-Fluorobenzyl), 7.59 (d, 2H, *J* = 8 Hz, $H_{2,6}$-4-Nitrophenyl), 8.21 (d, 2H, *J* = 8 Hz, $H_{3,5}$-4-Nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3159, 3041, 2922, 2850, 2729, 1693, 1580, 1516, 1346, 1298, 1174, 1066.

N-(5-(2-Nitrobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3g)

Yield: 25%, mp: 236 ºC, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.11 (s, 2H, -CH_2CO-), 4.82 (s, 2H, -CH_2-S-), 7.47 (m, 2H, 2-Nitrophenyl), 7.56 (m, 3H, 2-Nitrophenyl, 4-Nitrophenyl), 8.1 (d, 2H, *J* = 8 Hz, 2-Nitrophenyl), 8.19 (d, 2H, *J* = 8 Hz, 4-Nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3440, 2850, 2738, 1694, 1574, 1537, 1511, 1342, 1303, 1188, 960, 830, 704.

N-(5-(3-Nitrobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3h)

Yield: 45%, mp: 242 ºC, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.09 (s, 2H, -CH_2CO-), 4.51 (s, 2H, -CH_2-S-), 7.49 (t, *J* = 8 Hz, H_5-3-Nitrophenyl), 7.57 (d, *J* = 8 Hz, $H_{2,6}$-4-Nitrophenyl), 7.73 (d, *J* = 8 Hz, H_6-3-Nitrophenyl), 8.13 (d, *J* = 8 Hz, H_4-3-Nitrophenyl), 8.19 (d, *J* = 8 Hz, $H_{3,5}$-4-Nitrophenyl), 8.24 (s, H_2-4-Nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3440, 3318, 2920, 1690, 1560, 1523, 1349, 1306, 1172, 831, 809.

N-(5-(4-Nitrobenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3i)

Yield: 29%, mp: 258 ºC, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.42 (s, 2H, -CH_2CO-), 4.50 (s, 2H, -CH_2-S-), 7.55 (m, 4H, aromatic), 7.33 (m, 4H, aromatic). IR (KBr, cm^{-1}) ῡ: 3140, 3116, 3097, 2912, 1693, 1620, 1504, 1300, 1222, 1125, 1120, 940. MS (*m/z*, %): M^++2: 433 (10), M^++1: 432 (15), 431 (8), 352 (20), 268 (30), 191 (100), 189 (95), 149 (30), 130 (30), 82 (28), 70 (35), 55 (35).

N-(5-(3-Methoxybenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3j)

Yield: 24%, mp: 234-240 °C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 3.76 (s, 3H, -OCH_3), 4.11 (s, 2H, -CH_2CO-), 4.42 (s, 2H, -CH_2-S-), 6.79 (d, 1H, *J* = 8 Hz, H_6-3-Methoxybenzyl), 6.93 (m, 2H, $H_{2,4}$-3-Methoxybenzyl), 7.19 (t, 1H, *J* = 8 Hz, H_5-3-Methoxybenzyl), 7.91 (d, 2H, *J* = 8 Hz, $H_{2,6}$-4-Nitrophenyl), 8.17 (d, 2H, *J* = 8 Hz, $H_{3,5}$-4-Nitrophenyl), 13.27 (brs, NH). IR (KBr, cm^{-1}) ῡ: 3445, 2923, 2853, 1693, 1601, 1565, 1515, 1348, 1306, 1265, 1038, 830.

N-(5-(4-Methoxybenzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl)acetamide (3k)

Yield: 27%, mp: 220-227 °C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.78 (s, 3H, -OCH_3), 3.95 (s, 2H, -CH_2CO-), 4.40 (s, 2H, -CH_2S-), 6.82 (d, 2H, *J* = 8 Hz, 4-Methoxybenzyl), 7.29 (d, 2H, *J* = 8 Hz, 4-Methoxybenzyl), 7.57 (d, 2H, *J* = 8 Hz, 4-Nitrophenyl), 8.18 (d, 2H, *J* = 8 Hz, 4-Nitrophenyl), 12.9 (brs, NH). IR (KBr, cm^{-1}) ῡ: 3440, 3120, 2880, 1683, 1620, 1510, 1346, 1300, 1250, 1178, 1062, 829, 721. MS (*m/z*, %): M^++1: 417 (10), M^+: 416 (15), 396 (100), 369 (100), 368 (100), 353 (25), 344 (40), 340 (40).

N-(5-(Benzylthio)-1,3,4-thiadiazol-2-yl)-2-(4-nitrophenyl) acetamide (3l)

Yield: 41%, mp: 245-252 °C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.11 (s, 2H, -CH_2CO-), 4.45 (s, 2H, -CH_2-S-), 7.28 (m, 2H, benzyl), 7.35 (m, 3H, benzyl), 7.6 (d, 2H, *J* = 8 Hz, $H_{2,6}$-4-Nitrophenyl), 8.18 (d, 2H, *J* = 8 Hz, $H_{3,5}$-4-Nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3253, 3161, 3043, 2924, 2852, 1693, 1568, 1514, 1456, 1344, 1300, 1230, 829, 756.

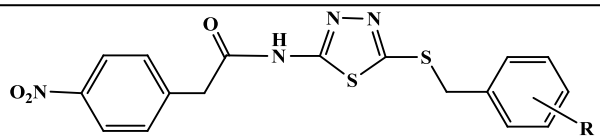
Cytotoxicity assay

Diverse derivatives of 1,3,4-thiadiazole(compounds 3a-3l) were tested for cytotoxic activity at 0.1-250 mcg/mL concentration in three human cancer cell lines of PC3 cell (prostate cancer), U87-C-531 (gliobalstoma) and MDA-MB-231 (breast cancer). Cells from different cell lines were seeded in 96-well plates at the density of 8000–10,000 viable cells per well and incubated for 48 hours to allow cell attachment. The cells were then incubated for another 48-96 hours (depends to cell cycle of each cell line) with various concentrations of compounds 3a-3l. Cells were then washed in PBS, and 20 μL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (5 mg/mL) were added to each well. An additional 4 hours of incubation at 37°C were done, and then the medium was discarded. Dimethyl sulfoxide (60 μL) was added to each well, and the solution was vigorously mixed to dissolve the purple tetrazolium crystals. The absorbance of each well was measured by a plate reader (Anthous 2020; Austria) at a test wavelength of 550 nm against a standard reference solution at 690 nm. The amount of produced purple formazan is proportional to the number of viable cells [23].

RESULTS AND DISCUSSION

All synthesized compounds were tested against three cancerous cell lines, PC3 (Prostate carcinoma), U87-C-531 (Glioblastoma) and MDA-MB-231 (Breast cancer) (Table 1). According to Table 1, compounds 3a-3l containing different substituents with electron withdrawing and electron donating properties rendered different anticancer properties. Totally four compounds demonstrated superior cytotoxic activity than imatinib as reference drug. Compound 3a with *ortho* substitution of chlorine showed more cytotoxic effect than imatinib against PC3 cell line. Moving the chlorine to the *meta* position caused a lower activity against PC3 cell line and a higher activity against MDA-MB-231 cell line (compound 3b), but did not show any acceptable potency against U87-C-531. Position *para* of the phenyl ring for chlorine was the worst position for all cell lines (compound 3c). Fluorine moiety exerted more cytotoxic effect against MDA-MB-231 cell line compared to other cell lines especially at *ortho* position (compound 3d). Increasing the electron withdrawing properties of the substituent (shifting from chlorine to fluorine) was detrimental for PC3 and U87-C-531 cell lines. Nitro substitution at position 3 (*meta*) of the phenyl ring caused anticancer properties of an acceptable towards the MDA-MB-231 cell line in compound 3h. Positions *para*, *ortho*, and *meta* were the best positions for exhibiting anticancer properties of nitro substituent against PC3, U87-C-531, and MDA-MB-231 respectively. Inserting a methoxy group as an electron donating moiety at positions *meta* and *para* was also explored. Methoxy at position *meta* of the phenyl ring (compound 3j) enhanced the anticancer

Table 1: IC_{50} (μM) for compound 3 with different R groups compared to imatinib.



Compound	R	PC3	U87-C-531	MDA-MB-231
3a	2-Cl	10.6	>250	74.5
3b	3-Cl	35.5	>250	27.3
3c	4-Cl	>250	154	>250
3d	2-F	>250	>250	10.3
3e	3-F	>250	>250	16.5
3f	4-F	93.5	>250	>250
3g	2-NO_2	124.5	62	>250
3h	3-NO_2	37	89	12.5
3i	4-NO_2	17.7	84	188
3j	3-OCH_3	>250	>250	11.3
3k	4-OCH_3	>250	71	>250
3l	H	>250	182	>250
Imatinib	-	16	15	16

activity against MDA-MB-231 cell line. Inserting a phenyl ring without any moiety (compound 3l) did not cause any significant effect.

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