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Synthesis of novel 5-substituted phenyl-3-(p-isopropylphenyl)-1-phenylformazan and their biological activities

Ayşe ŞAHİN YAĞLIOĞLU[1,*], **Hülya ŞENÖZ**[2]
[1]Department of Chemistry, Faculty of Science, Çankırı Karatekin University, Çankırı, Turkey
[2]Department of Chemistry, Faculty of Science, Hacettepe University, Ankara, Turkey

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Abstract: Novel 1-substituted 3-(p-isopropylphenyl)-5-phenylformazans (**3a**–**g**) were synthesized and characterized by elemental analysis, ^{1}H NMR, and FT-IR techniques and UV-visible spectroscopy. Antiproliferative activities of **3a**–**g** against HeLa and C6 cells were determined using the BrdU cell proliferation ELISA assay. 5-Florouracil was used as the positive control. The effects of substituents (–H, –CH_3, and –I) and their positions (*ortho*, *meta*, and *para*) on the antiproliferative activities were evaluated. The results of the assay indicate that I substituent exhibited higher activity against the cells at the *meta* and *para* positions than –CH_3 and –H substituents. **3a**–**g** exhibited both high antiproliferative activities against C6 cells and noncytotoxicity. **3a**–**g** may be anticancer drug candidates.

Key words: Formazan, diazo coupling, antiproliferative activity, cytotoxic activity, HeLa cell, C6 cell

1. Introduction

Formazans are of wide interest because of their biological and pharmacological activities such as antiviral,[1,2] antifungal,[3] antifertility,[4] antiinflammatory,[5] antitubercular,[6] and antimicrobial[7,8] activities. These compounds are used for the determination of trace elements,[9] preparation of metal complexes,[10,11] and synthesis of heterocyclics.[12] Formazans and their metal complexes are used as dyes for wool, stable to sunlight and water.[13,14] This feature is the cause of the increasing interest in the chemistry of formazans.

Depending on the nature of the substituents, formazans show a variety of biological activities. When the literature is examined, it is seen that many derivatives of formazan compounds, such as nitro, methoxy, chlorine, fluorine, methyl, bromine, and hydroxy, are synthesized.[15−18] However, no formazans substituted with isopropylphenyl in the 3-position and methyl and iodophenyl in the 5-position derivatives and the cancer activities of these derivatives have not been found. Moreover, from these studies, methyl derivatives of formazan compounds were found.[15−18] However, the main formazan skeleton is not the same and cancer activity studies have not been found. Thus, we aimed to synthesize new formazans by methyl or iodo groups at the o,m,ppositions of the phenyl ring 1 and isopropyl group at the p position of the phenyl ring 3. It was also aimed to investigate the effects of the type and the location of the substituents on their antiproliferative activities against HeLa and C6 cell lines.

*Correspondence: aysesahin1@gmail.com

2. Results and discussion

2.1. Chemical results

There are distinctive routes proposed for the synthesis of formazans in the literature.[19] The known approach is the coupling reaction of hydrazones with diazonium salts.[1] Formazans **3a**–**g** were prepared[20] by the coupling reactions of 1-(4-isopropylbenzylidene)-2-phenylhydrazine (**1**)[21] with the benzene diazonium chlorides of substituted anilines (**2a**–**g**) in a basic medium at 0 to –5 °C. The reaction is shown in Figure 1.

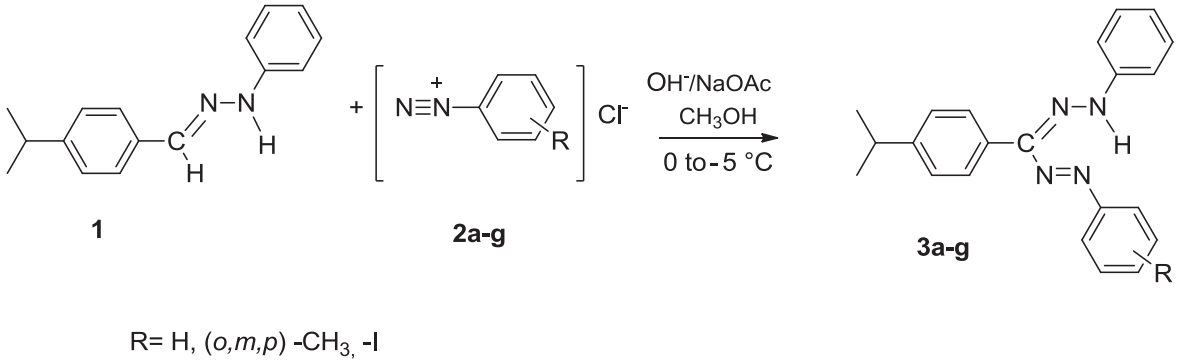

Figure 1. Synthesis of formazans.

The formazans form intramolecular hydrogen bonds between the lone pair of N(1) and H atom on N(5). Thus, the formazans have a chelated hydrogen-bridge structure[1] (Figure 2).

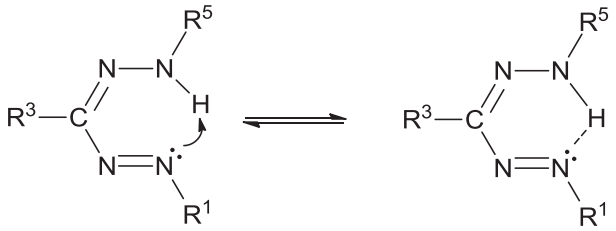

Figure 2. Molecular chelation.

The FTIR spectra of formazans **3a**–**g** showed characteristic bands in the ranges 3053–3081 cm^{-1} (N–H), 2958–3030 cm^{-1} (Ar–H), 1588–1607 cm^{-1} (C=C aromatic), 1502–1522 cm^{-1} (C=N), and 1445–1498 cm^{-1} (N=N). Weak N–H stretching bands were observed in the range 3053–3081 cm^{-1}, and the NH signal of the compounds in the ^{1}H NMR spectra was observed in the low field at $\sim$15 ppm. This indicates that the structure has intramolecular hydrogen bonds. In addition, the lower values of the C=N and N=N bands show a chelate structure.[1,13]

The ^{1}H NMR data show that the signals of aromatic hydrogens of **3a**–**g** appeared in the range 6.84–8.10 ppm. The aromatic H peaks were distributed in a broad range because each of the three formazan rings has a different electron density. **3a**–**g** exhibited sharp NH signals in the downfield region between 15.09 and 15.40 ppm indicative of an intramolecular hydrogen bond, which decreases electron density around the proton and thus moves the proton absorption to the lower field. Formazans **3b**–**d** exhibited a singlet peak for Ar–CH_3 protons in the range 2.42–2.45 ppm. The doublet peak for the CH_3 protons of isopropyl groups appeared in the range 1.20–1.35 ppm and the multiplet peak for the CH proton appeared in the range 2.88–3.01 ppm.

2.2. Antiproliferative activity

The IC_{50} and IC_{75} values of the compounds against the cells are shown in Table 1.

Table 1. The IC_{50} and IC_{75} values of the compounds against HeLa and C6 cell lines.

Compound name	HeLa cell line		C6 cell line	
	IC_{50}	IC_{75}	IC_{50}	IC_{75}
3a	< 5	36.65	< 5	< 5
3b	27.49	52.50	< 5	28.07
3c	31.73	57.94	25.69	54.20
3d	10.90	43.25		26.34
3e	58.82	87.71	44.38	69.46
3f	< 5	< 5	< 5	< 5
3g	< 5	11.48	< 5	< 5

2.2.1. Effects of different substituents at the *ortho*-position of compounds on their antiproliferative activities against HeLa and C6 cell lines

According to the antiproliferative activities of **3a**, **3b**, **3e**, and 5-FU against the HeLa cell line, **3a** and **3b** exhibited stronger antiproliferative activities than 5-FU at high concentrations (75–100 μM). **3e** exhibited weaker antiproliferative activity than 5-FU and **3a** and **3b**. The activities of all the compounds increased with the increase in concentration (Figure 3A).

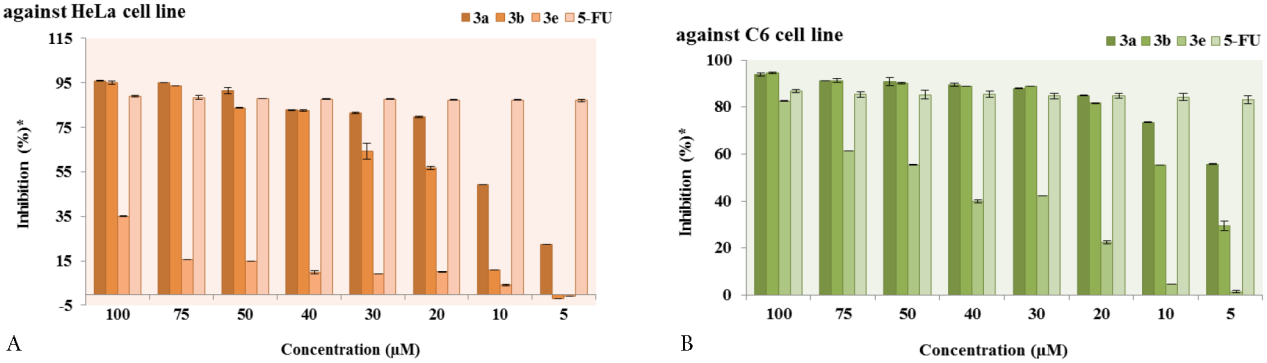

Figure 3. (A) Effects of antiproliferative activities of different *ortho* substituent of **3a**, **3b**, **3e** against HeLa (A) and C6 (B) cell lines. *Each substance was tested at least twice in triplicate against cell lines. Data show the average of two individual experiments ($P < 0.01$).

The potency of the inhibitions (at 100 μM) against HeLa cells decreased in the following order: **3a** $\approx$ **3b**$>$ 5-FU $>$ **3e**, i.e. o-H $\approx$ o-CH_3 $>$ 5-FU$>$ $o-I$.

According to the antiproliferative activities of **3a**, **3b**, **3e**, and 5-FU against the C6 cell line, **3a** and **3b** exhibited stronger antiproliferative activities than 5-FU at 30–100 μM concentrations. **3e** exhibited almost the same antiproliferative activity compared to 5-FU at 100 μM concentration. The activities of all the compounds increased with the increase in concentration, and the results show that especially **3e** exhibited a cell-selective effect against the C6 cell line (Figure 3B).

At 100 μM concentration, the activities of compounds against C6 cell line decreased in the following order: **3a** $\approx$ **3b** $>$ 5-FU $>$ **3e**, i.e. o-H $\approx$ o-CH_3 $>$ 5-FU $>$ o-I.

2.2.2. Effects of different substituents at the *meta*-position of compounds on their antiproliferative activities against HeLa and C6 cell lines

According to the antiproliferative activities of **3a**, **3c**, **3f**, and 5-FU against the HeLa cell line, **3a** at 50–100 μM concentrations and **3f** at 20–100 μM concentrations exhibited higher activities than 5-FU. **3c** exhibited lower antiproliferative activity than 5-FU and **3a** and **3f**. The activities of all the compounds increased with the increase in concentration (Figure 4A). At 100 μM concentration, the activity of compounds against HeLa cells decreased in the following order: **3f** $>$ **3a** $>$ 5-FU $>$ **3c**, i.e. $m-I > m-H > 5-FU > m$-CH_3.

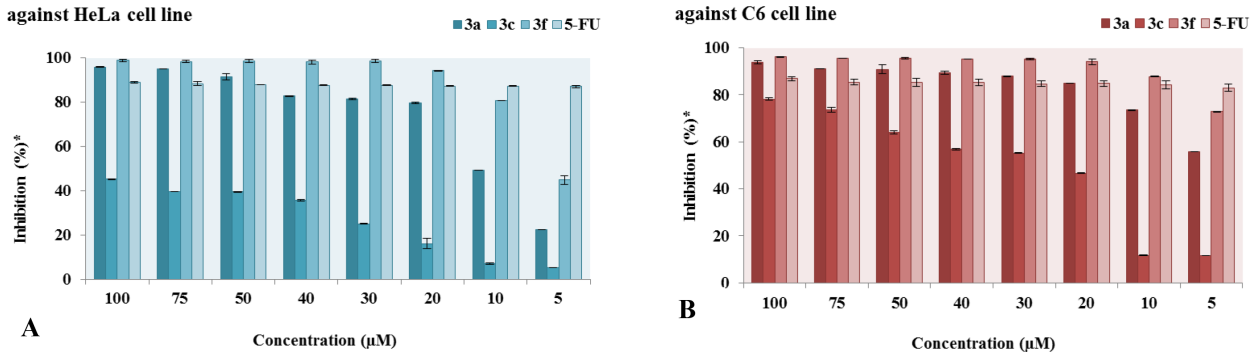

Figure 4. Effects of antiproliferative activities of different *meta* substituent of **3a**, **3c**, **3f** against HeLa **(A) and C6 (B)** cell lines. *Each substance was tested at least twice in triplicate against cell lines. Data show the average of two individual experiments ($P < 0.01$).

According to the antiproliferative activities of **3a**, **3c**, **3f**, and 5-FU against the C6 cell line, **3a** and **3f** exhibited stronger antiproliferative activities than 5-FU at 20–100 μM concentrations. **3c** exhibited considerably higher antiproliferative activity than 5-FU at 100 μM concentration. The activities of all the compounds increased with the increase in concentration, and the results show that especially **3c** exhibited a cell-selective effect against the C6 cell line (Figure 4B). At 100 μM concentration, the activities of compounds against the C6 cell line decreased in the following order: **3f** $>$ **3a** $>$ 5-FU $>$ **3c**, i.e. m-I $> m$-H $>$ 5-FU $> m$-CH_3.

2.2.3. Effects of different substituents at the *para*-position of compounds on their antiproliferative activities against HeLa and C6 cell lines

According to the antiproliferative activities of **3a**, **3d**, **3g**, and 5-FU against the HeLa cell line, **3a**, **3d**, and **3g** exhibited stronger activities than 5-FU at 50–100 μM concentrations. The activities of all the compounds increased with the increase in concentration (Figure 5A). At 100 μM concentration, the activities of compounds against HeLa cells decreased in the following order: **3g** $>$ **3a** $\approx$ **3d** $>$ 5-FU, i.e. p-I $> p$-H $\approx p$-CH_3 $>$ 5-FU.

According to the antiproliferative activities of **3a**, **3d**, **3g**, and 5-FU against the C6 cell line, **3a**, **3d**, and **3g** exhibited stronger antiproliferative activities than 5-FU at 75–100 μM concentrations. The activities of all the compounds increased with the increase in concentration (Figure 5B). At 100 μM concentration, the activities of compounds against the C6 cell line decreased in the following order: **3g** $>$ **3a** $>$ **3d** $>$ 5-FU, i.e. p-I $> p$-H $> p$-CH_3 $>$ 5-FU.

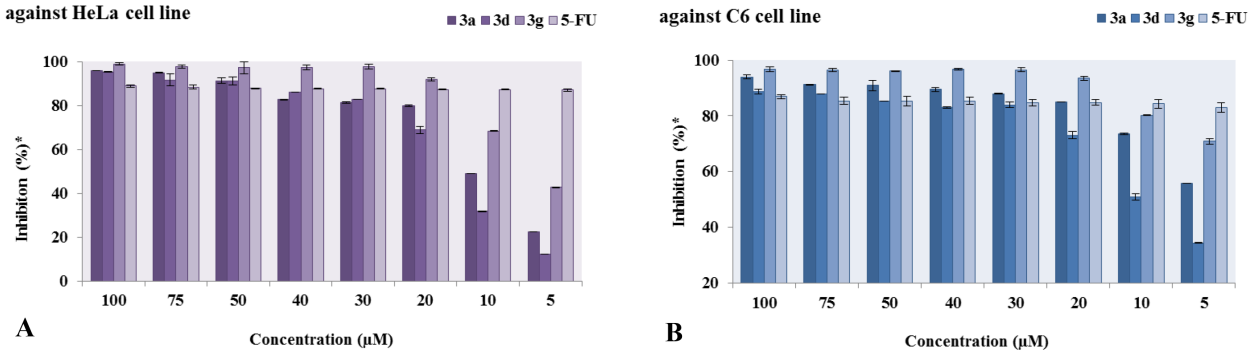

Figure 5. Effects of antiproliferative activities of different *para* substituent of **3a**, **3d**, **3g** against HeLa (A) and C6 (B) cell lines. *Each substance was tested at least twice in triplicate against cell lines. Data show the average of two individual experiments ($P < 0.01$).

2.2.4. Effects of the same substituents at the *ortho*-, *meta*-, and *para*-positions of compounds on their antiproliferative activities against HeLa and C6 cell lines

According to the antiproliferative activities of **3b**, **3c**, **3d**, and 5-FU against the HeLa cell line, **3b** and **3d** exhibited stronger antiproliferative activities than 5-FU at 75–100 μM concentrations. The activities of all the compounds increased with the increase in concentration (Figure 6A).

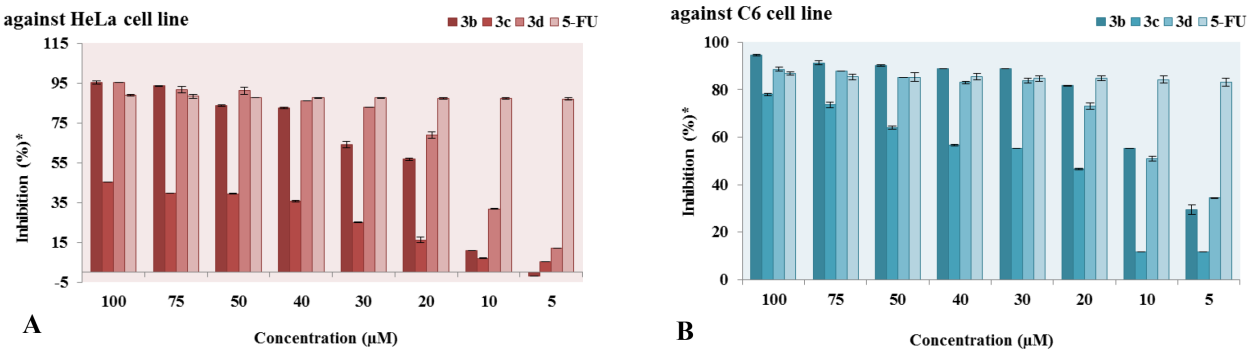

Figure 6. Effects of antiproliferative activities of the different *ortho*-, *meta*-, and *para*-CH_3 substituents of **3b**, **3c**, **3d** against HeLa (A) and C6 (B) cell lines. *Each substance was tested at least twice in triplicate against cell lines. Data show the average of two individual experiments ($P < 0.01$).

At 100 μM concentration, the activities of compounds against HeLa cells decreased in the following order: **3b** $\approx$ **3d** $>$ 5-FU $>$ **3c**, i.e. *o*-CH_3 $\approx$ *p*-CH_3 $>$ 5-FU $>$ *m*-CH_3.

According to the antiproliferative activities of **3b**, **3c**, **3d**, and 5-FU against the C6 cell line, **3b** and **3d** exhibited stronger antiproliferative activities than 5-FU at 75–100 μM concentrations. The activities of all the compounds increased with the increase in concentration (Figure 6B).

At 100 μM concentration, the activities of compounds against the C6 cell line decreased in the following order: **3b** $>$ **3d** $>$ 5-FU $>$ **3c**, i.e. *o*-CH_3 $\approx$ *p*-CH_3 $>$ 5-FU $>$ *m*-CH_3.

According to the antiproliferative activities of **3e**, **3f**, **3g**, and 5-FU against the HeLa cell line, **3f** and **3g** exhibited stronger antiproliferative activities than 5-FU at 20–100 μM concentrations. The activities of all the compounds increased with the increase in concentration (Figure 7A). At 100 μM concentration, the activities of compounds against HeLa cells decreased in the following order: **3f** $\approx$ **3g** $>$ 5-FU $>$ **3e**, i.e. *m*-I $\approx$ p-I $>$ 5-FU $>$ o-I.

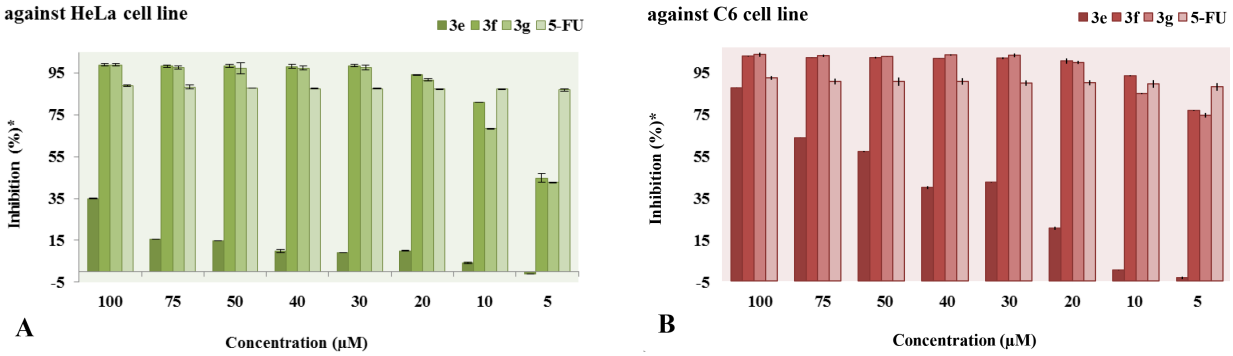

Figure 7. Effects of antiproliferative activities of the different *ortho*-, *meta*-, and *para*-I substituents of **3e**, **3f**, **3g** against HeLa (A) and C6 (B) cell lines. *Each substance was tested at least twice in triplicate against cell lines. Data show the average of two individual experiments ($P < 0.01$).

According to the antiproliferative activities of **3e**, **3f**, **3g**, and 5-FU against the C6 cell line, **3f** and **3g** exhibited stronger antiproliferative activities than 5-FU at 20–100 μM concentrations. The activities of all the compounds increased with the increase in concentration (Figure 7B).

At 100 μM concentration, the activities of compounds against the C6 cell line decreased in the following order: **3f** $\approx$ **3g** $>$ 5-FU $>$ **3e**, i.e. m-I $\approx$ p-I $>$ 5-FU $>$ o-I.

2.3. Cytotoxic activity

C6 cells were used to determine the cytotoxic activities (%) of compounds. The test results are shown in Table 2. At 100 μM concentration, **3a**–**g** were found to be nontoxic compared to 5-FU.

Table 2. Cytotoxic activities of **3a**–**3g** and 5-FU.

Sample name	Cytotoxicity (%)
3a	0
3b	0
3c	0
3d	0
3e	0
3f	0
3g	0
5-FU	6

3. Conclusion

3a, **3b**, **3d**, **3f**, and **3g** exhibited higher inhibitory effects against both HeLa and C6 cancer cells than 5-FU, the standard compound. **3c** and **3e** exhibited remarkable activities against C6 cells compared to 5-FU. In addition, **3c** and **3e** showed a cell-selective effect against the C6 cell line. According to the cytotoxic activity results against C6 cells, **3a**–**3g** are less toxic at the investigated highest dose than 5-FU. These values are very promising. **3a**–**g** exhibited both high antiproliferative activities and noncytotoxicity. The results show the importance of **3a**–**g**.

4. Experimental

The UV-visible spectra of formazans were recorded using a UV-1700 Pharma spectrophotometer. The FTIR spectra were recorded using a Nicolet IS10-FTIR spectrometer, and the ^{1}H NMR spectra were recorded using a Bruker 400-MHz FT-NMR spectrometer. The elemental analyses were carried out using a LECO-CHNS- 932 elemental analyzer.

4.1. General procedure for the synthesis of formazans (3a–g)

Formazans **3a**–**g** were prepared following the literature procedures.[19–21] 1-(4-Isopropylbenzylidene)-2-phenylhydrazine (1) (5 mmol) was dissolved in methanol (35 mL), and a buffer solution consisting of sodium hydroxide (0.44 g), sodium acetate (0.66 g), and 35 mL of methanol was added to this solution. Diazonium chlorides of aniline or substituted anilines (**2a**–**g**) (5 mmol) were added to the hydrazine solution at -5 °C. The mixture was stirred at -5 °C for 2 h and then left for 2 days. **3a**–**g** were recrystallized from ethanol.

3-(*p*-Isopropylphenyl)-1,5-diphenylformazan (3a): Yield (1.29 g, 75%), Clear red-black crystals, mp 143 °C. FTIR: 3077, 3039, 2959, 1595, 1498, 1303, 1224, 838, 686 cm^{-1}. λ_{max}/nm (CH_2Cl_2): 300, 491. ^{1}H NMR ($CDCl_3$) δ: 1.29–1.31 (d, 6H, CH_3), 2.95–2.99 (m, 1H, CH), 7.25–7.32 (m, 4H, ArH), 7.43–7.47 (t, 4H, ArH), 7.67–7.69 (d, 4H, ArH), 8.04–8.06 (d, 2H, ArH), ArH), 15.33 (s, 1H, NH). Anal. Calcd. (%) for $C_{22}H_{22}N_4$: C 77.16, H 6.47, N 16.36. Found: C 77.38, H 6.20, N 16.05.

3-(*p*-Isopropylphenyl)-1-phenyl-5-(*o*-tolyl)formazan (3b): Yield (1.38 g, 77%), Clear red crystals, mp 141 °C; FTIR: 3074, 3030, 2954, 2930, 1719,1607, 1504, 1463, 1357, 1035, 753 cm^{-1}. λ_{max}/nm (CH_2Cl_2): 305, 500. ^{1}H NMR ($CDCl_3$) δ: 1.20–1.31 (d, 6H, CH_3), 2,45 (s, 3H, CH_3), 2.94–3.01 (m, 1H, CH), 7.14–7.18 (t, 1H, ArH), 7.26–7,33 (m, 5H, ArH), 7.45–7.49 (t, 2H, ArH), 7.74–7.76 (d, 2H, ArH), 7.99–8.01 (d, 1H, ArH), 8.08–8.10 (d, 2H, ArH), 15.40 (s, 1H, NH). Anal. Calcd. (%) for $C_{23}H_{24N4}$: C 77.50, H 6.79, N 15.72. Found: C 77.25, H 6.45, N 15.40.

3-(*p*-Isopropylphenyl)-1-phenyl-5-(*m*-tolyl)formazan (3c): Yield (1.25 g, 70%), rusty brown crystals, mp 129 °C; FTIR: 3081, 3018, 2974, 1605, 1522, 1458, 1218, 767, 670 cm^{-1}. λmax/nm (CH_2Cl_2): 300, 493. ^{1}H NMR ($CDCl_3$) δ: 1.20–1.31 (d, 6H, CH_3), 2.45 (s, 3H, CH_3), 2.94–3.01 (m, 1H, CH), 7.09–7.11 (d, 1H, ArH), 7.24–7.36 (m, 4H, ArH), 7.43–7.50 (m, 4H, ArH), 7.67–7.66 (d, 2H, ArH), 8.04–8.06 (d, 2H, ArH), 15.33 (s, 1H, NH). Anal. Calcd. (%) for $C_{23}H_{24}N_4$: C 77.50, H 6.79, N 15.72. Found: C 77.28, H 6.53, N 15.51.

3-(*p*-Isopropylphenyl)-1-phenyl-5-(*p*-tolyl)formazan (3d): Yield (1.30 g, 73%), rusty brown crystals, mp 124 °C; FTIR: 3078, 3012, 2959, 2933, 1595, 1516, 1451, 1215, 1035, 741 cm^{-1}. λmax/nm (CH_2Cl_2): 304, 492. ^{1}H NMR ($CDCl_3$) δ: 1.29–1.35 (d, 6H, CH_3), 2,42 (s, 3H, CH_3), 2.94–3.00 (m, 1H, CH), 7.15–7.19 (t, 1H, ArH), 7.26–7.32 (m, 4H, ArH), 7.39–7.43 (t, 2H, ArH), 7.56–7.58 (d, 2H, ArH), 7.68–7.70 (d, 2H, ArH), 8.04–8.06 (d, 2H, ArH), 15.31 (s, 1H, NH). Anal. Calcd. (%) for $C_{23}H_{24}N_4$: C 77.50, H 6.79, N 15.72. Found: C 77.32, H 6.45, N 15.35.

5-(*o*-Iodophenyl)-3-(p-isopropylphenyl)-1-phenylformazan (3e): Yield (1.69 g, 73%), clear red crystals, mp 137 °C. FTIR: 3060, 3020, 2957, 2924, 2865, 1597, 1502, 1445, 1346, 1219, 1175, 1009, 836, 753, 685 cm^{-1}. λ_{max}/nm (CH_2Cl_2): 302, 494. ^{1}H NMR ($CDCl_3$) δ: 1.25–1.32 (d, 6H, CH_3), 2.92–3.01 (m, 1H, CH), 6.93–6.97 (t, 1H, ArH), 7.25–7.26 (d, 3H, ArH), 7.45–7.49 (t, 3H, ArH), 7.86–7.88 (d, 1H, ArH), 8.06–8.08 (d, 2H, ArH), 15.09 (s, 1H, NH). Anal. Calcd. (%) for $C_{22}H_{21}N_4I$: C 56.42, H 4.52, N 11.97. Found: C 56.11, H 4.45, N 11.71.

5-(*m*-Iodophenyl)-3-(*p*-isopropylphenyl)-1-phenylformazan (3f): Yield (1.58 g, 68%), dark clear brown crystals, mp 110 °C. FTIR: 3058, 3015, 2950, 2926, 1588, 1460, 1340, 1212, 1167, 1101, 912, 820, 780 cm^{-1}. λ_{max}/nm (CH_2Cl_2): 486. ^{1}H NMR ($CDCl_3$) δ: 1.23–1.34 (d, 6H, CH_3), 2.89–2.95 (m, 1H, CH), 6.83–6.98 (t, 1H, ArH), 7.29–7.32 (d, 3H, ArH), 7.40—7.49 (t, 3H, ArH), 7.68–7.78 (d, 1H, ArH), 8.02–8.07 (d, 2H, ArH), 15.09 (s, 1H, NH). Anal. Calcd. (%) for $C_{22}H_{21}N_4I$: C 56.42, H 4.52, N 11.97. Found: C 56.20, H 4.33, N 11.52.

5-(*p*-Iodophenyl)-3-(*p*-isopropylphenyl)-1-phenylformazan (3g): Yield (1.62 g, 70%), clear red crystals, mp 120 °C. FTIR: 3053, 3015, 2957, 2926, 1596, 1503, 1484, 1454, 1397, 1355, 1225, 1180, 1197, 1053, 920, 828, 794, 688 cm^{-1}. λ_{max}/nm (CH_2Cl_2): 492. ^{1}H NMR ($CDCl_3$) δ: 1.25–1.35 (d, 6H, CH_3), 2.88–2.95 (m, 1H, CH), 6.84–6.87 (t, 1H, ArH), 7.09–7.11 (d, 3H, ArH), 7.20–7.27 (m, 3H, ArH), 7.57–7.59 (d, 1H, ArH), 8.01–8.03 (d, 2H, ArH), 15.20 (s, 1H, NH). Anal. Calcd. (%) for $C_{22}H_{21}N_4I$: C 56.42, H 4.52, N 11.97. Found: C 56.05, H 4.28, N 11.63.

4.2. Biological activities

Cell proliferation ELISA, BrdU (colorimetric) kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany). 5-Florouracil was used as the positive control, and other chemicals were purchased from Sigma, Merck, and Roche.

4.2.1. Preparation of stock solutions

The samples and 5-fluorouracil (5-FU) were dissolved in dimethyl sulfoxide (final concentration is below 1%) and diluted with Dulbecco's modified Eagle medium (DMEM). HeLa and C6 cell lines were cultured in DMEM.

4.2.2. Cell proliferation assay

The antiproliferative activities of the compounds against HeLa and C6 cell lines were evaluated by proliferation BrdU ELISA assay.[22–25]

4.2.3. Lactate dehydrogenase (LDH) leakage assay

The LDH leakage assay was carried out following the method reported by Eser et al.[21]

4.2.4. Statistical analysis

The results show the means ± SD of nine measurements. Differences among the groups were determined by ANOVA. P values of <0.01 were considered significant.

4.2.5. Determination of IC50 and IC75 values

IC50 and IC75 values were calculated using the ED50 plus v1.0 program.

Acknowledgments

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