The Antiproliferative effects of subtituents in formazan derivatives aganist HeLa and C6 cell line

Formazan türevlerindeki sübstitüentlerin HeLa ve C6 hücrelerine karşı antiproliferatif etkileri

Research Article

Ayse Şahin Yağlıoğlu[1,*] and Hülya Şenöz[2]
[1]Department of Chemistry, Faculty of Science, Çankırı Karatekin University, 18100 Çankırı, Turkey
[2]Department of Chemistry, Faculty of Science, Hacettepe University, 06800 Ankara, Turkey

ABSTRACT

Antiproliferation activity of TPF and 1-substituted phenyl-3-(p-methoxycarbonyl)phenyl-5-phenylformazans were determined against HeLa an C6 cell line using BrdU cell proliferataion ELISA assay. Cisplatin and 5-florouracil were used as standarts. The activities of samples and standarts were investigated on eight concentations. The effects of substituents and substituents positions on antiproliferation activities were examined. Assay experiments indicated that o- OCH_3, m-CH_3 and p-I displayed maximum activity against HeLa while o- NO_2, m-I and also p-I were active against C6.

Key Words

Antiproliferative activity, HeLa, C6, formazan derivatives, substituent effect.

ÖZET

TPF ve 1-sübstitüe fenil-3-(p-metoksikarbonil)fenil-5-fenilformazanların antiproliferatif aktiviteleri HeLa ve C6'ya karşı BrdU hücre proliferasyon ELİSA yöntemi ile belirlendi. Standart olarak sisplatin ve 5-florourasil kullanıldı. Örneklerin ve standartların aktiviteleri sekiz farklı derişimde incelenmiştir. Sübstitüentlerin ve sübstitüent konumlarının antiproliferatif aktivite üzerine etkileri araştırıldı. Deneysel çalışmalar sonucunda, o- OCH_3, m-CH_3 ve p-I sübstitüentlerinin HeLa hücresine karşı, o- NO_2, m-I ve p-I sübstitüentlerinin ise C6 hücresine karşı maksimum etkiye sahip olduğu belirlendi.

Anahtar Kelimeler

Antiproliferatif aktivite , HeLa, C6, formazan, sübstitüent etkisi

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Correspondence to: A. Ş. Yağlıoğlu, Department of Chemistry, Faculty of Science, Çankırı Karatekin University, 18100 Çankırı, Turkey

Tel: +90376 218 1123/ 5003 Fax:+90376 218 1031 E-Mail: aysesahin1@gmail.com

INTRODUCTION

Formazans are characterized by their intense colors, ranging from cherry red to a deep purplish black and contain the characteristic chain of atoms (R)N=N-C(R)=N-NH(R). These compounds are used as analytical reagents, photochromic and thermochromic materials in redox reactions and in the synthesis of heterocyclic compounds due to their unique acidity, alkalinity, isomerization, tautomerization and pi conjugation properties.

In addition, formazans have exhibited a variety of biological activities. A literature survey has revealed that various substituted formazans are known to possess antimicrobial[1,2], analgesic [3], anti-inflammatory [3-4], antitubercular [5], anticancer [6] and anti HIV [6] activities.

In this work, we studied the antiproliferative activities of a number of 1-substituted phenyl-3-(p-methoxycarbonyl)phenyl-5-phenyl formazans (**1-14**) (Figure 1).

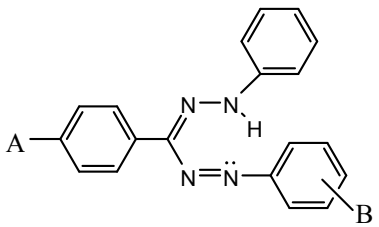

Figure 1. A= H, p-$COOCH_3$; B= H,(o,m,p)- NO_2,-CH_3,-OCH_3, -I

MATERIALS AND METHODS

Chemicals

The antiproliferative activities chemicals used in the present study were in analytical grade and obtained from Sigma–Aldrich, Merck and Roche.

Cell culture and cell proliferation assay

The antiproliferative activities tests was carried out following the procedure of Demirtas et al. [7]. HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells were grown in Dulbecco's modified eagle's medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum (Sigma, Germany) and 2% (v/v) PenStrep solution (Sigma, Germany) at 37°C in a 5% CO_2 humudified atmosfer. For proliferation assay, cells were plated in 96-well culture plates (COSTAR, Corning, USA) at a densitiy of 30.000 cells per well. Vehicle (DMSO), 5-Florouracil, cisplatin and several the samples in various concentrations (5-100 μg/mL) were added to each well. Cells were than incubated for overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to manufacturer's procedure . Briefly, cells were pulsed with BrdU labeling reagent for 4 h followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1:100 dilution of anti- BrdU-POD for 1.30 h at room temperature. The amount of cell proliferation was assessed by determining the A450 nm of the culture media after addition of the substrate solution by using a microplate reader (Ryto, China). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. All assays were repeated at least twice using against HeLa and C6 cells.

Table 1. IC_{50} and IC_{75} values of the most effective compounds.

	C6 Cell Line			HeLa Cell Line		
	5	**13**	**14**	**5**	**13**	**14**
IC50	22.94	-	37.65	49.01	-	46.47
IC750	52.03	31.45	61.04	68.82	33.58	63.98

Percentage of inhibition of cell proliferation was calculated as follows: $[1-(A_{treatments} / A_{vehicle\ control}) \times 100$.

Statistical Analysis

The results of investigation in vitro are the means ± SEM of six measurements for each cell type. Differences between treatment groups were tested way ANOVA and p values of <0.01 were considered significant.

RESULTS

Effects to antiproliferative activities of the same substituents at ortho (o-), meta (m-) and para (p-) positions of the compounds aganist HeLa cell line

Formazans **1-14** used in this study were prepared by the coupling reaction of phenylhydrazones

and diazonium salts of aniline or -NO_2, -CH_3, -OCH_3, -I, substituted anilines. Synthesis and spectral properties of these formazan have been explained in the previous study [8].

Antiproliferation activity of compounds (**1-14**) were determined against HeLa cell line using BrdU cell proliferataion ELISA assay. Cisplatin and 5-florouracil were used as standarts. The activities of samples and standarts were investigated on eight concentations (5, 10, 20, 30, 40, 50, 75 and 100 μg/mL). The IC50 and IC75 values of the most effective compounds aganist HeLa were shown at Table 1.

The compound (**2**) has higher antiproliferative activity than the compound (**1**) (Figure 2A). In other words, the effects to antiproliferative activities of $COOCH_3$ to be found at para positions (p-) were showed better than H substituent to be at the same positions. In addition to, either the compound (**1**) or the compound (**2**) have quite weak activities to compare with cisplatin and 5-florouracil. However, in this compounds activities were shown to increase depending to increasing concentration (Figure 2A). The potency of inhibition was 5-FU> Cisplatin> compound (**2**) > compound (**1**), in other words, 5-FU> Cisplatin> p-$COOCH_3$> p-H.

According to Figure 2B, the compound (**5**) have higher antiproliferative activities than the compound (**3**) and compound (**4**) and in the compound (**5**) activities was shown to increase depending to increasing concentration (Figure 2B). At the same time, the antiproliferative activities of the compound (**3**) and compound (**4**) were observed to have rather near activities. Also, the standart compounds were established more activities than compounds (**3**, **4**, **5**) [except at 75 and 100 μg/mL concentration of compound (**5**)]. In respect of this results, the effect to antiproliferative activities of substituents was 5-FU> Cisplatin> p-NO_2> o-NO_2 ~ m-NO_2.

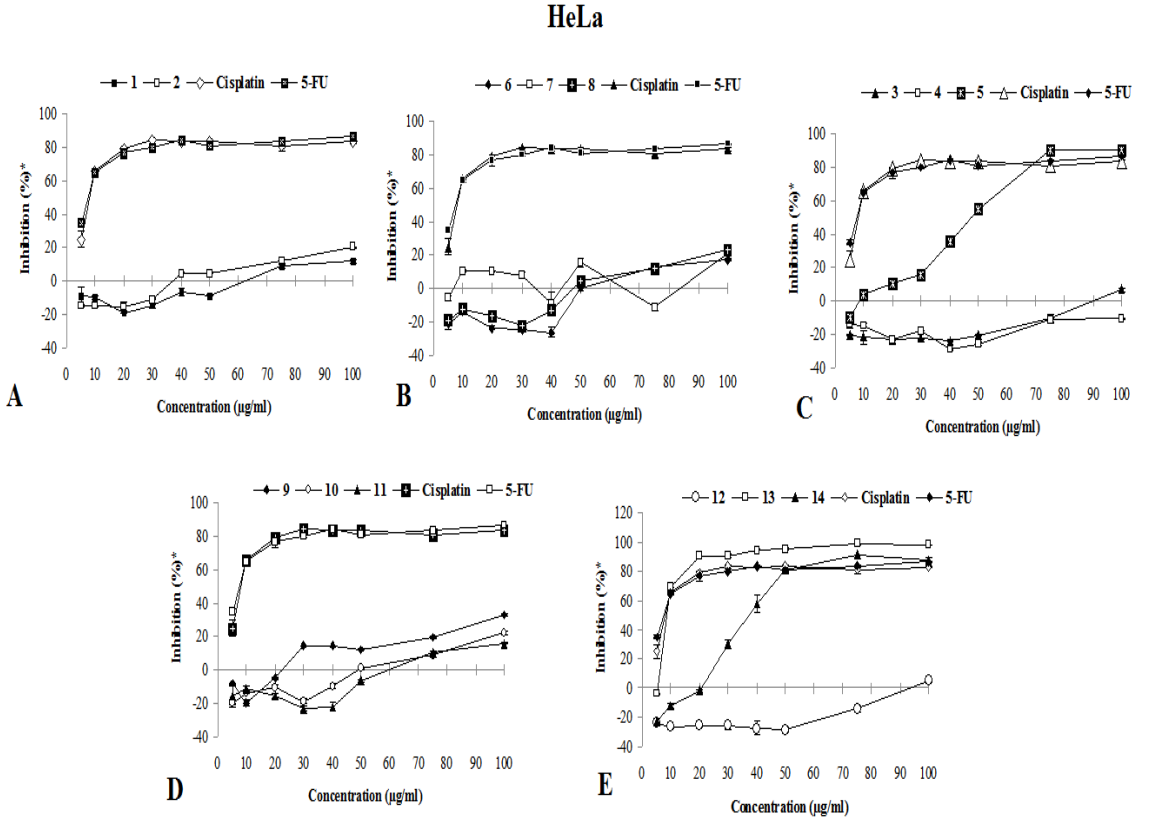

Figure 2. (A) Effects of antiproliferative activities of diffrent para substituent of **1-2** aganist HeLa cell line. (B) Effects of antiproliferative activities of the ortho, meta and para -NO_2 substituents of **3-5** aganist HeLa cell line. (C) Effects of antiproliferative activities of ortho, meta and para -CH_3 substituent of **6-8** aganist HeLa cell line. (D) Effects of antiproliferative activities of ortho, meta and para -OCH_3 substituents of **9-11** aganist HeLa cell line. (E) Effects of antiproliferative activities of ortho, meta and para -I substituents of **12-14** aganist HeLa cell line.* Each substance was tested at least twice in triplicates against cell lines. Data show avarage of 2 individual experiments ($p<0.01$).

Although the compound (**7**) was shown better antiproliferative activities than compounds (**6**) and (**8**) [except at 75 and 100 μg/mL concentration], this three compounds have weak activities to compare with standarts compounds (Figure 2C). In addition to, the antiproliferative activities of the compound (**6**) and compound (**8**) were observed to have very nearly the same activities. The potency of inhibition was 5-FU› Cisplatin› compound (**7**) › compound (**6**) ~ compound (**8**), in other words, 5-FU› Cisplatin› m-CH_3› p-CH_3 ~ o-CH_3.

According to Figure 2D, the compound (**9**) has more strong antiproliferative activities than compounds (**10**) and (**11**). Morever, the antiproliferative activities of the compounds (**10**) and (**11**) were determined to be very nearly same. The antiproliferative of the three compounds were very weak to compare with 5-FU and cisplatin. The potency of inhibition was 5-FU› Cisplatin› compound (**9**)› compound (**10**)~ compound (**11**), in other words, 5-FU› Cisplatin› o-OCH_3› m-OCH_3 ~ p-OCH_3.

The compound (**13**) has the higher activities than either the compounds (**12**) and (**14**) and standarts compounds. In addition to, the compound (**14**) has more strong activities than the compound (**12**) and in the compounds activities was shown to increase depending to increasing concentration (Figure 2E). Morever, the antiproliferative activities of the compound (**14**) was determined to be the higher than standarts compounds at 75 and 100 μg/mL concentration. The potency of inhibition was compound (**13**) › 5-FU › Cisplatin › compound (**14**) › compound (**12**), in other words, m-I› 5-FU › Cisplatin› p-I› o-I.

Effects of antiproliferative activities of the different substituents at o-positions of the compounds aganist HeLa cell line.

The compounds (**3**, **6**, **9**, **12**) were shown more weak antiproliferative activities to compare with standarts compounds. Between this compounds, compound (**9**) has better activities than the compounds (**3**, **6**, **12**). In all of the compounds activities was shown to increase depending to increasing concentration (Figure 3A). The potency of inhibition was 5-FU › Cisplatin › compound (**9**) › compound (**6**)› compound (**3**) ~ compound (**12**), in other words, 5-FU › Cisplatin› o-OCH_3› o-CH_3 › o-NO_2 ~ o-I.

Effects of antiproliferative activities of the diffrent substituents at m-positions of the compounds aganist HeLa cell line.

The compound (**13**) were shown more strong antiproliferative activities to compare with standarts compounds and the compounds (**4**, **7**, **10**) . In all of the compounds activities was shown to increase depending to increasing concentration

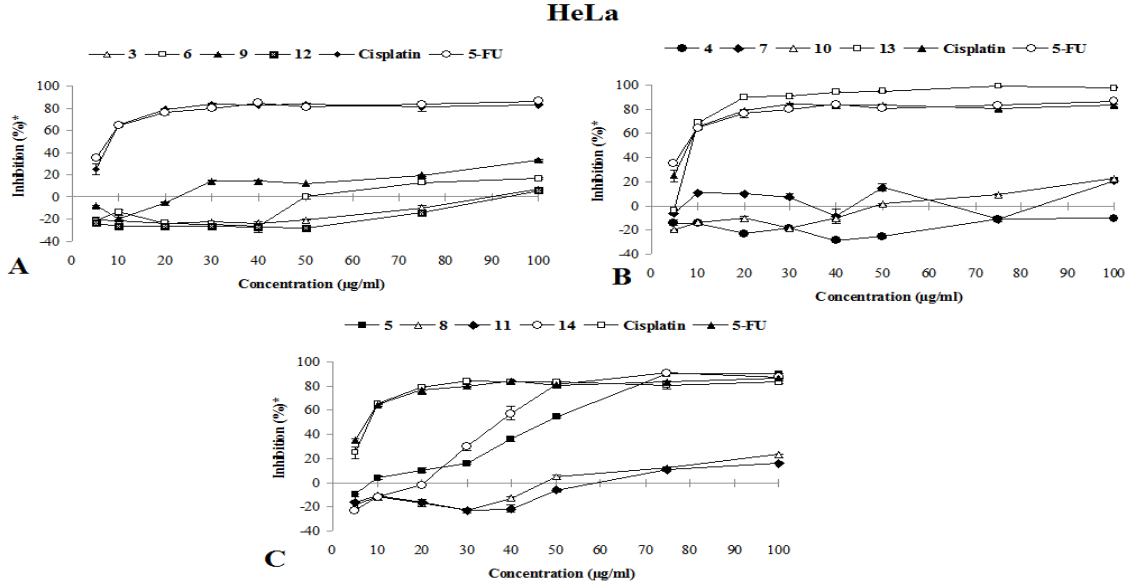

Figure 3. (A) Effects of antiproliferative activities of the different ortho substituents of **3, 6, 9, 12** aganist HeLa cell line. (B) Effects of antiproliferative activities of the different meta substituents of **4, 7, 10, 13** aganist HeLa cell line. (C) Effects of antiproliferative activities of the different para substituents of **5, 8, 11, 14** aganist HeLa cell line. * Each substance was tested at least twice in triplicates against cell lines. Data show avarage of 2 individual experiments ($p<0.01$).

(Figure 3B). The potency of inhibition was compound (**13**) > 5-FU > Cisplatin > compound (**7**) > compound (**10**) > compound **(4),** in other words, m-I > 5-FU > Cisplatin> m-CH_3> m-OCH_3 > m-NO_2.

Effects of antiproliferative activities of the different substituents at p-positions of the compounds aganist HeLa cell line.

Although the compounds (**5**, **8**, **11**, **14**) were shown more weak antiproliferative activities to compare with standarts compounds, the compound (**14**) (at 50-100 μg/mL) and the compound (**5**) (at 75-100 μg/mL) have the similar activities to compare with standarts compounds. In all of the compounds activities was shown to increase depending to increasing concentration (Figure 3C). The potency of inhibition was 5-FU > Cisplatin > compound (**14**) > compound (**5**) > compound **(8)** > compound (**11**), in other words, 5-FU > Cisplatin> p-I> p-NO_2 > p-CH_3> p-OCH_3.

Effects to antiproliferative activities of the same substituents at o, m, p-positions of the compounds aganist C6 cell line

Antiproliferation activity of compounds (**1-14**) were determined against C6 cell line using BrdU cell proliferataion ELISA assay. Cisplatin and 5-florouracil were used as standarts. The activities of samples and standarts were investigated on eight concentations (5, 10, 20, 30, 40, 50, 75 and 100 μg/mL). The IC50 and IC75 values of the most effective compounds aganist C6 were shown at Table 1.

The compound (**2**) has higher antiproliferative activities than the compound (**1**) (Figure 4A). In other words, the effects to antiproliferative activities of $COOCH_3$ to be found at para positions (p-) were showed better than H substituent to be at the same positions. In addition to, either the compound (**1**) or the compound (**2**) have quite weak activities to

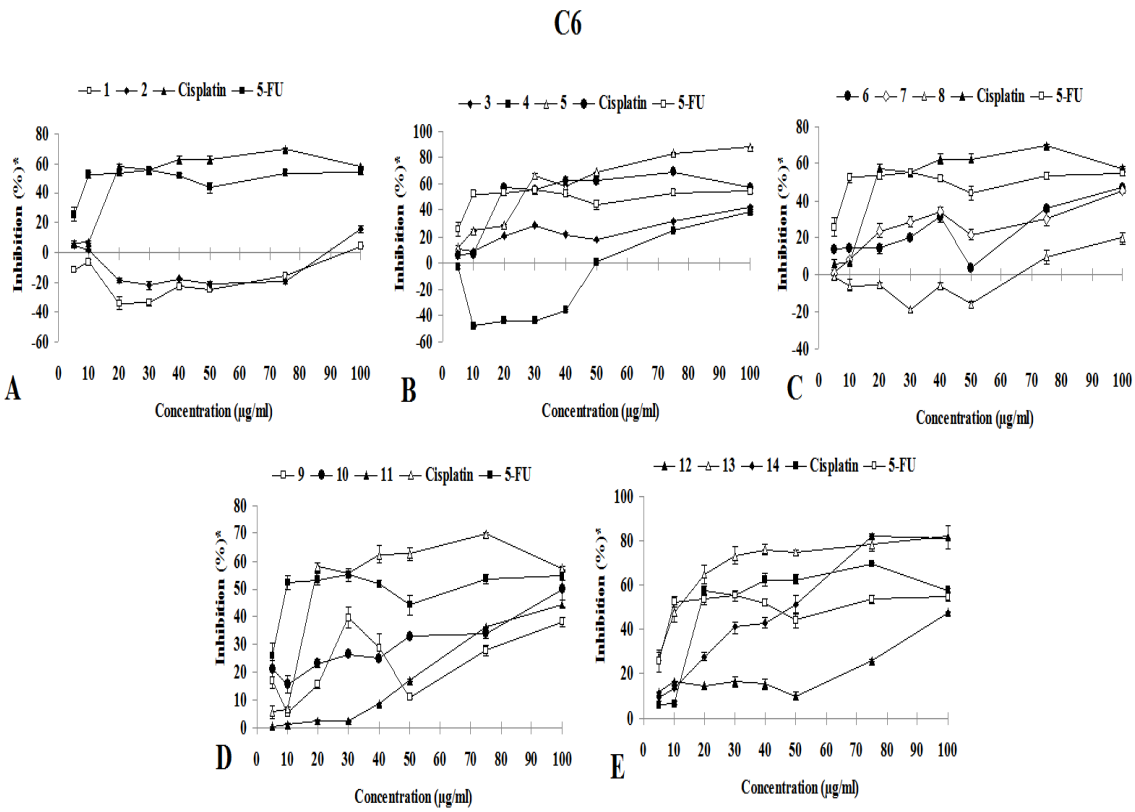

Figure 4. (A) Effects of antiproliferative activities of the different para substituent of **1-2** aganist C6 cell line. (B) Effects of antiproliferative activities of the different ortho, meta and para -NO_2 substituents of **3-5** aganist C6 cell line. (C) Effects of antiproliferative activities of the ortho, meta and para -CH_3 substituents of **6-8** aganist C6 cell line. (D) Effects of antiproliferative activities of ortho, meta and para -OCH_3 substituent of **9-11** aganist C6 cell line. (E) Effects of antiproliferative activities of ortho, meta and para -I substituents of **12-14** aganist C6 cell line.* Each substance was tested at least twice in triplicates against cell lines. Data show avarage of 2 individual experiments ($p<0.01$).

compare with cisplatin and 5-florouracil. However, in this compounds activities was shown to increase depending to increasing concentration (Figure 4A). The potency of inhibition was 5-FU> Cisplatin> compound (**2**) > compound (**1**), in other words, 5-FU> Cisplatin> p-$COOCH_3$> p-H.

According to Figure 4B, the compound (**5**) (above at 20 μg/mL concentration) have higher antiproliferative activities than the compound (**3**) and compound (**4**) and standarts compounds. All of this compounds was shown to increase depending to increasing concentration at the antiproliferative activities (Figure 4B). At the same time, the antiproliferative activities of the compound (**3**) have especially higher than antiproliferative activities of the compound (**4**) at the low concentration (5-50 at μg/mL concentration). were observed to have rather near activities. Also, the standart compounds were established more activities than compounds (**3**, **4**). The potency of inhibition was compound (**5**) > Cisplatin > 5-FU> compound (**3**) > compound (**4**), in other words, p-NO_2> Cisplatin > 5-FU> o-NO_2 > m-NO_2.

The antiproliferative activities compound (**7**) and compound (**6**) were observed to have very nearly the same activities. The compounds (**6**, **7**, **8**) have weak activities to compare with standarts compounds (Figure 4C). In addition to, antiproliferative activities of the compound (**8**) were determined as worst to compare with the compounds (**6**, **7**) and standarts compounds. The potency of inhibition was Cisplatin> 5-FU> compound (**6**) compound (**7**) compound (**8**), in other words, Cisplatin > 5-FU > o-CH_3 ~ m-CH_3 > p-CH_3.

According to Figure 4D, the compound (**10**) has more strong antiproliferative activities than compounds (**9**) and (**11**). The antiproliferative of the three compounds were very weak to compare with 5-FU and cisplatin. The potency of inhibition was Cisplatin> 5-FU> compound (**10**) > compound (**11**) > compound (**9**), in other words, Cisplatin > 5-FU> m-OCH_3> p-OCH_3 > o-OCH_3.

The compound (**13**) has the higher activities than either the compounds (**12**) and (**14**) and standarts compounds. In addition to, at the compounds activities was shown to increase depending to increasing concentration (Figure 4E). Morever, the antiproliferative activities of the compound (**14**) were determined to be the higher than standarts compounds at 75 and 100 μg/mL concentration. The potency of inhibition was compounds (**13**) > Cisplatin >5-FU> compound (**14**) > compound (**12**), in other words, m-I> 5-FU > Cisplatin> p-I> o-I.

Effects of antiproliferative activities of the different substituents at o-positions of the compounds aganist C6 cell line.

The compounds (**3**, **6**, **9**, **12**) were shown more weak antiproliferative activities to compare with standarts compounds. In all of the compounds activities was shown to increase depending to increasing concentration (Figure 5A). The potency of inhibition was Cisplatin > 5-FU > compound (**3**) ~ compound (**6**) ~ compound **(9)** ~ compound **(12),** in other words, Cisplatin > 5-FU > o-NO_2 ~ o-CH_3 ~ o-OCH_3 ~ o-I.

Effects to antiproliferative activities of the different substituents at m-positions of the compounds aganist C6 cell line.

The compound (**13**) was shown more strong antiproliferative activities to compare with standarts compounds and the compounds (**4**, **7**, **10**) . In all of the compounds activities was shown to increase depending to increasing concentration (Figure 5B). The potency of inhibition was compound (**13**) > Cisplatin > 5-FU > compound (**10**) > compound (**7**) > compound **(4),** in other words, m-I > Cisplatin > 5-FU > m-OCH_3 > m-CH_3> > m-NO_2.

Effects to antiproliferative activities of the different substituents at para positions of compounds aganist C6 cell line.

The compounds (**5**, **14**) were shown better antiproliferative activities to compare with standarts compounds (The compounds (**5**, **14**) have very nearly the same activities to with with standarts) and other compounds. In all of the compounds activities was shown to increase depending to increasing concentration (Figure 5C). The potency of inhibition was compound (**5**) > compound (**14**) > Cisplatin > 5-FU > compound (**11**) > compound **(8),** in other words, p-NO_2> p-I> Cisplatin > 5-FU > p-OCH_3 > p-CH_3.

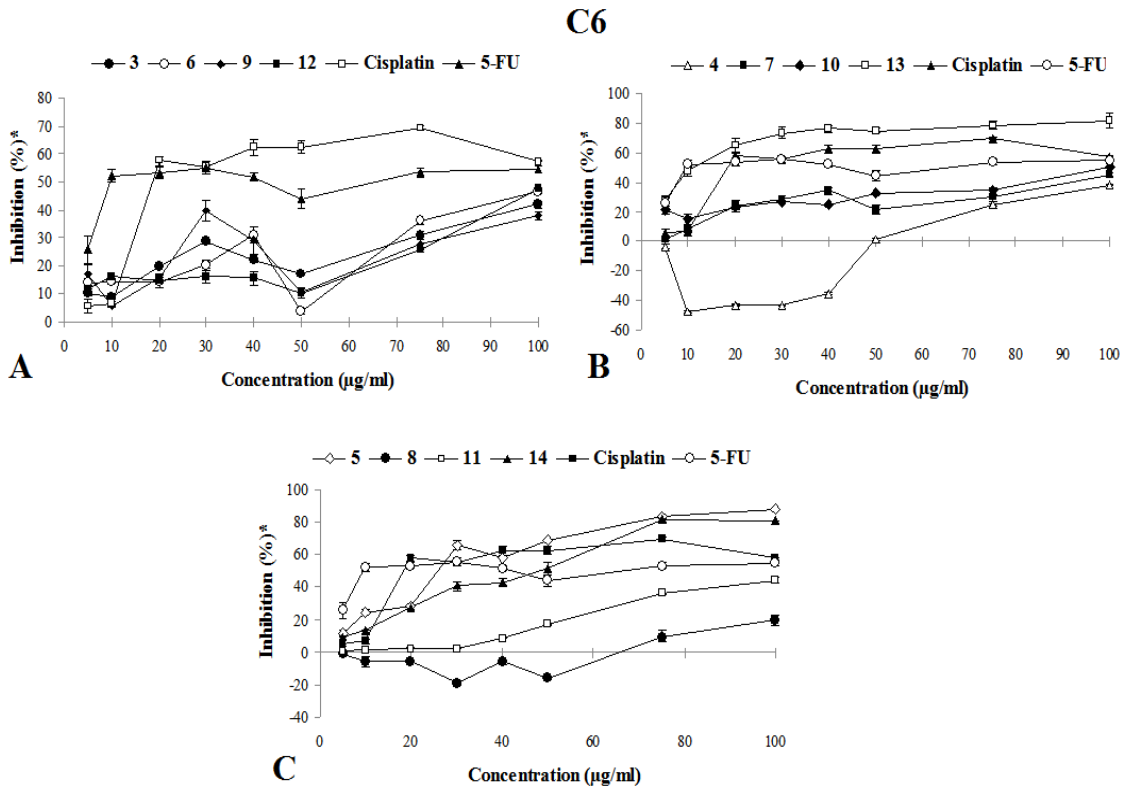

Figure 5. (A) Effects of antiproliferative activities of the different ortho substituent of **3, 6, 9, 12** aganist C6 cell line. (B) Effects of antiproliferative activities of the different meta substituent of **4, 7, 10, 13** aganist C6 cell line. (C) Effects of antiproliferative activities of the different para substituent of **5, 8, 11, 14** aganist C6 cell line. * Each substance was tested at least twice in triplicates against cell lines. Data show avarage of 2 individual experiments ($p<0.01$).

DISCUSSION

Effects to antiproliferative activities of ortho (o-), meta (m-) and para (p-) substituent of compounds were determined aganist HeLa and C6 cell line. According to HeLa cell line, the potency of inhibition was 5-FU > Cisplatin > p-$COOCH_3$ > p-H; 5-FU > Cisplatin > p-NO_2 > o-NO_2 ~ m-NO_2; 5-FU > Cisplatin > m-CH_3 > p-CH_3 ~ o-CH_3; 5-FU > Cisplatin > o-OCH_3 > m-OCH_3 ~ p-OCH_3; m-I > 5-FU > Cisplatin > p-I > o-I. According to C6 cell line, 5-FU > Cisplatin > p-$COOCH_3$ > p-H; p-NO_2 > Cisplatin > 5-FU > o-NO_2 > m-NO_2; Cisplatin > 5-FU > o-CH_3 ~ m-CH_3 > p-CH_3; Cisplatin > 5-FU > m-OCH_3 > p-OCH_3 > o-OCH_3; m-I > 5-FU > Cisplatin > p-I > o-I.

However, effects of antiproliferative activities of different ortho, meta and para substituent of compounds were investigated aganist HeLa and C6 cell line. In respect of HeLa, the potency of inhibition was 5-FU > Cisplatin > o-OCH_3 > o-CH_3 > o-NO_2 ~ o-I; m-I > 5-FU > Cisplatin > m-CH_3 > m-OCH_3 > m-NO_2; 5-FU > Cisplatin > p-I > p-NO_2 > p-CH_3 > p-OCH_3. In respect of C6, the potency of inhibition was Cisplatin > 5-FU > o-NO_2 ~ o-CH_3 ~ o-OCH_3 ~ o-I; m-I > Cisplatin > 5-FU > m-OCH_3 > m-CH_3 > m-NO_2; p-NO_2 > p-I > Cisplatin > 5-FU > p-OCH_3 > p-CH_3.

In sum, the maximum effects to antiproliferative activities of the different substituents (between -NO_2, -CH_3, -OCH_3 and -I substituent) were -OCH_3 for ortho, -CH_3 for meta and -I for para aganist HeLa. The same effects were -NO_2 for ortho and para, -I for meta aganist C6. The maximum effects to antiproliferative activities of the same substituents at different positions (between o-, m- and p-) were ortho for -OCH_3, meta for -CH_3 and para for -I aganist HeLa. The same effects were ortho and para for -NO_2, meta for -I aganist C6.

Conflicts of interest

No potential conflicts of interest were disclosed.

ACKNOWLEDGMENTS

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