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Short communication

Synthesis and antitumor activity of ureas containing pyrimidinyl group

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ABSTRACT

A novel series of ureas containing pyrimidinyl group were designed and synthesized. Some of the prepared compounds showed potential cytotoxicity against several human cancer cell lines. From the structure-activity relationships we may conclude that introduction of a sulfide bridge between phenyl and pyrimidinyl rings would be critical for their biological activities.

1. Introduction

Cancer is a major worldwide health problem. Although there has been progress in the development of treatment and prevention for cancer, this disease remains the second major cause of death in the world. Still, the successful treatment of cancer remains a challenge in the 21st century, and there is a need to search for newer and safer anticancer agents that have a broader spectrum of cytotoxicity to tumor cells.

Urea derivatives have been used for the treatment of a wide range of solid tumors [1–6]. Urea-based prodrugs have been reported as candidates for melanocyte-directed enzyme prodrug therapy (MDEPT), in which they release the drug upon exposure to tyrosinase [7]. Some related urea derivatives also have been reported as protein tyrosine kinases (PTKs) inhibitors, and have become an important class of potential anticancer drugs [8]. For these reasons, the synthesis of urea and their functionalized derivatives is of high interest.

Bioisosterism is an effective way to design bioactive compounds [9]. The pyrimidinyl group is a highly efficient pharmacophore and is widely used in pesticide and drug molecular design [10–12]. We wanted to investigate whether there would be some new beneficial properties if pyrimidinyl group was combined in the urea derivatives. In order to find more potent urea antitumor agents, we designed and synthesized novel ureas containing the pyrimidinyl group. The bioactivities of the new compounds were evaluated.

2. Chemistry

Synthesis of these novel compounds was carried out in a straightforward manner.

Compound **3** could be synthesized starting from 4-aminophenol or 4-aminobenzenethiol **1** and 4,6-dimethy-2-(methylsulfonyl) pyrimidine **2** in presence of K_2CO_3 in DMF [13]. Treatment of equimolar amounts of substituted isocyanates with compound **3** in dry CH_2Cl_2 afforded the corresponding urea derivatives (**4a**–**4l**) (Scheme 1). The preparations are summarized in Table 1. The chemical structures of the compounds were confirmed by ^{1}H NMR, ^{13}C NMR and elementary analysis and the results are presented in Section 6.

3. Pharmacology

The in vitro antitumor activity of the synthesized compounds against four cancer cell lines, including KB (oral carcinoma cell), CNE2 (nasopharyngeal carcinoma cell), MGC-803 (gastric carcinoma cell), and MCF-7 (breast adenocarcinoma cell), were assayed by MTT method. 5-FU was used as the reference drug. Table 2 reported the IC_{50} (μM) values (concentration required to achieve 50% inhibition of the tumor growth) of the tested compounds and the standards. With respect to the in vitro cytotoxic activity expressed in Table 2, most of the prepared compounds showed potential cytotoxicity against several human cancer cell lines.

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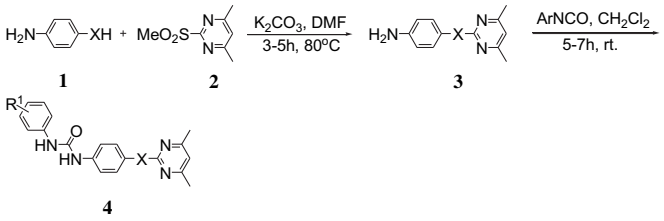


Scheme 1. General synthetic route for compounds **4a-l**

4. Results and discussion

4.1. Nuclear magnetic resonance spectral studies

The evidence for the formation of the compounds was obtained from ^{1}H NMR and ^{13}C NMR spectra, which provide diagnostic tools for the positional elucidation of the protons. Assignments of the signals are based on the chemical shifts and intensity patterns. The ^{1}H NMR spectrum of all the compounds showed singlet for two (2 NH) protons atδ (8.20–9.48), another singlet for one (pyrimidine-H) proton atδ (6.70–7.00) and a singlet for two (2 CH_3) proton atδ (2.25–2.32). Thus on the basis of the above data the products have been characterized as urea derivatives.

^{13}C NMR spectra of all the compounds were taken in DMSO-d_6 and the signal obtained further confirmed the proposed structures. All the compounds showed a signal at (151.9–152.6) ppm due to (C=O) of urea derivatives. Urea derivative showed a signal at (169.4–170.6) ppm due to (N–C=N). Methyl carbon displayed a signal at (23.3–23.4) ppm of urea derivatives. The characteristic peaks observed within the ^{13}C NMR spectra of urea derivatives are given in Section 6.

4.2. In vitro cytotoxicity

The results indicated that the pyrimidinyl group introduced to urea's structure was useful for the improvement of antitumor activity. For the compounds without pyrimidinyl group displayed very weak antitumor activity against the tumor cells. As seen from Table 2, most of prepared compounds showed potential cytotoxicity against KB and CNE2 cancer cell lines, and some compounds showed moderate cytotoxicity against MGC-803 cancer cell line. However, most of the compounds showed weak cytotoxicity against MCF-7 cancer cell line. In conclusion, the cytotoxicities of the tested compounds against KB and CNE2 were higher than MCF-7 cell line, reflecting the selectivity for a particular human oral carcinoma and human nasopharyngeal carcinoma cancer cell types.

The cytotoxicities of the resulting urea derivatives appeared to be related to the nature of the substituent group. Some compounds displayed more or similar potent cytotoxic activities against certain cancer cell lines in comparison with 5-FU. For KB cell line, the compound **4k** showed the best inhibitory activity with IC_{50} 10.2 μM. Introduction of a sulfide bridge between phenyl and pyrimidinyl rings resulted in higher activity than did an ether link. For example, among **4i**–**4l** bearing a sulfide bridge moieties, the IC_{50} values of them are 20.0 μM, 32.1 μM, 10.2 μM and 15.5 μM respectively, which were superior to **4c**–**4f** bearing an ether link moieties with the IC_{50} values of 28.9 μM, 48.4 μM, 25.4 μM and 31.8 μM respectively. For CNE2 cell line, introduction of a sulfide bridge between phenyl and pyrimidinyl rings also resulted in higher activity than did an ether link. The compound **4k** showed the best inhibitory activity with IC_{50} 13.5 μM.

For MGC-803 cell line, most of compounds with an ether link displayed very weak antitumor activity against the tumor cells. However, the compound **4i** showed the best inhibitory activity with IC_{50} 13.8 μM. For MCF-7 cell lines, most of the compounds showed low activity.

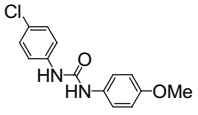
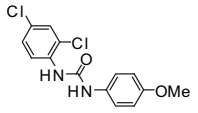
Still, the change of R^1, also had a little influence on their activity. For example, 4-Cl substituted compounds (**4e** and **4k**) demonstrated the most strong cytotoxic activities against KB with IC_{50} 25.4 and 10.2 μM respectively.

Table 1
The preparation of ureas **4a-l**

Compound	X	R^1	Yield(%)[a]	Mp (°C)
4a	O	H	86	187.3–190.0
4b	O	3-Me	86	213.3–214.8
4c	O	4-OCF_3	76	208.8–210.5
4d	O	4-F	85	219.6–220.4
4e	O	4-Cl	83	225.8–226.7
4f	O	2,4-diCl	83	220.3–222.1
4g	S	H	86	211.4–213.2
4h	S	3-Me	80	196.1–197.8
4i	S	4-OCF_3	82	218.8–219.5
4j	S	4-F	84	207.9–210.0
4k	S	4-Cl	81	228.3–229.4
4l	S	2,4-diCl	86	246.8–248.5

[a] Isolated yields.

Table 2
Cytotoxic activity of urea compounds against human tumor cells.

Compound	In vitro cytotoxicity IC_{50}(μM)			
	KB[a]	CNE2[a]	MGC-803[a]	MCF-7[a]
4a	>50	–[b]	–	–
4b	>50	–	–	–
4c	28.9 ± 0.6	30.8 ± 2.3	>50	>50
4d	48.4 ± 1.4	46.9 ± 1.4	>50	>50
4e	25.4 ± 2.1	20.4 ± 1.6	>50	>50
4f	31.8 ± 2.1	38.9 ± 1.3	35.4 ± 1.6	47.8 ± 1.1
4g	25.7 ± 1.1	22.0 ± 1.1	40.1 ± 2.2	>50
4h	47.8 ± 1.2	>50	25.3 ± 1.5	37.0 ± 2.3
4i	20.0 ± 1.4	22.8 ± 1.6	13.8 ± 0.9	41.7 ± 1.4
4j	32.1 ± 2.7	34.1 ± 1.3	>50	>50
4k	10.2 ± 0.6	13.5 ± 1.1	26.1 ± 1.6	35.3 ± 2.5
4l	15.5 ± 0.3	23.2 ± 1.7	>50	>50
	>50	>50	–	>50
	>50	>50	>50	>50
5-FU	11.9 ± 1.1	13.1 ± 1.5	12.6 ± 1.6	15.2 ± 2.6

[a] KB cells were the drug sensitive human oral carcinoma cells, CNE2 cells were the drug sensitive human nasopharyngeal carcinoma cells, MGC-803 cells were the drug sensitive human gastric carcinoma cells and MCF-7 cells were the drug sensitive human breast adenocarcinoma cells.
[b] not tested.

5. Conclusion

In summary, we have designed and synthesized a novel series of ureas derivatives. Some of the prepared compounds showed potential cytotoxicity against several human cancer cell lines. Some compounds displayed more or similar potent cytotoxic activities against cancer cell lines in comparison with 5-FU. These findings have encouraged us to continue the development and testing of novel ureas and to conduct further studies to investigate SAR and their mechanisms of action.

6. Experimental protocols

6.1. General procedures

^{1}H NMR spectra were recorded at 600 MHz, in $CDCl_3$ or DMSO-d_6 solution on a Varian Mercury-Plus 600 spectrometer and chemical shifts were recorded in parts per million (ppm) with TMS as the internal reference. MS spectra were determined using a TraceMS 2000 organic mass spectrometry, and signals were given in *m*/*z*. Melting points were taken on a Buchi B-545 melting point apparatus. Elemental analysis (EA) was measured on a Vario ELIII CHNSO elemental analyzer. All commercially available solvents and reagents were used as supplied by Acros Organics unless otherwise stated. The silica gel (200–300 meshes) for flash column chromatography was from Qingdao Marine Chemical Factory in China.

6.2. Procedure for preparation of compound **3a**,**3b**

A solution of 4,6-dimethyl-2-(methylsulfonyl)pyrimidine (18 mmol), 4-aminophenol or 4-aminobenzenethiol (18 mmol) and K_2CO_3 (36 mmol) in DMF (50 mL) was stirred at 100 °C for 2 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with water and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (PE:EA = 2:1) to give the compound **3a**,**3b**.

6.3. General procedure for preparation of compounds **4a**–**4l**

Compound **3** (1 mmol) was dissolved in 15 mL dichloromethane. Temperature was maintained at 0 °C, then substituted phenyl isocyanate (1 mmol) was added drop wise with constant stirring. After 1 h, formation of white solid and the resultant solid product was filtered, washed with little ethanol and dried under vacuum. Recrystallization with ethanol afforded the desired solid urea.

6.4. Structural data

6.4.1. 4-(4,6-Dimethylpyrimidin-2-yloxy)benzenamine (**3a**) [14]

Yellow solid, yield: 95%, mp 139.1–140.3 °C; ^{1}H NMR ($CDCl_3$, 600 MHz): ^{1}H NMR ($CDCl_3$, 600 MHz): 2.38 (s, 6H, CH_3), 6.68 (s, 1H, CH), 6.70 (d, 2H, J = 8.4 Hz, ArH), 6.99 (d, 2H, J = 8.4 Hz, ArH). MS (EI, 70 eV): *m*/*z* 215 (M^+, 63), 200 (40), 119 (100), 91 (40), 69 (41).

6.4.2. 4-(4,6-Dimethylpyrimidin-2-ylthio)benzenamine (**3b**) [14]

Yellow solid, yield: 75%, mp 162.2–164.3 °C; ^{1}H NMR ($CDCl_3$, 600 MHz): 2.33 (s, 6H, CH_3), 6.66 (s, 1H, CH), 6.70 (d, 2H, J = 8.4 Hz, ArH), 7.38 (d, 2H, J = 8.4 Hz, ArH). MS (EI, 70 eV): *m*/*z* 231 (M^+, 100), 216 (27), 124 (29), 80 (27), 67 (28).

6.4.3. 1-(4-(4,6-Dimethylpyrimidin-2-yloxy)phenyl)-3-phenylurea (**4a**)

White solid, yield: 86%, mp 187.3–190.0 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.32 (s, 6H, CH_3), 6.94–6.98 (m, 1H, ArH), 6.99 (s, 1H, CH), 7.07 (d, 2H, J = 9.0 Hz, ArH), 7.27–7.29 (m, 2H, ArH), 7.46–7.47 (m, 4H, ArH), 8.68 (s, 1H, NH), 8.70 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 115.2, 118.1, 119.3, 121.8, 122.0, 128.7, 136.5, 139.8, 147.5, 152.6, 164.6, 169.4; Anal. Calcd. for $C_{19}H_{18}N_4O_2$ (%): C 68.25, H 5.43, N 16.76; Found: C 68.47, H 5.07, N 16.61.

6.4.4. 1-(4-(4,6-Dimethylpyrimidin-2-yloxy)phenyl)-3-m-tolylurea (**4b**)

White solid, yield: 86%, mp 213.3–214.8 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.28 (s, 3H, CH_3), 2.32 (s, 6H, CH_3), 6.79 (s, 1H, ArH), 6.99 (s, H, CH), 7.06–7.30 (m, 5H, ArH), 7.46 (d, 2H, J = 7.8 Hz, ArH), 8.59 (s, 1H, NH), 8.67 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 21.3, 23.3, 115.2, 115.3, 118.7, 119.2, 121.8, 122.5, 128.7, 136.5, 138.0, 139.7, 147.5, 152.6, 164.7, 169.4; Anal. Calcd. for $C_{20}H_{20}N_4O_2$ (%): C 68.95, H 5.79, N 16.08; Found: C 69.03, H 5.53, N 16.16.

6.4.5. 1-(4-(4,6-Dimethylpyrimidin-2-yloxy)phenyl)-3-(4-(trifluoromethoxy)phenyl)urea (**4c**)

White solid, yield: 76%, mp 208.8–210.5 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.32 (s, 6H, CH_3), 7.00 (s, 1H, CH), 7.08 (d, 2H, J = 9.0 Hz, ArH), 7.29 (d, 2H, J = 9.0 Hz, ArH), 7.47 (d, 2H, J = 9.0 Hz, ArH), 7.57 (d, 2H, J = 9.6 Hz, ArH), 8.76 (s, 1H, NH), 8.90 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 115.2, 119.4, 119.6, 121.1, 121.8, 122.0, 136.3, 139.0, 142.6, 147.7, 152.6, 164.7, 169.4; Anal. Calcd. for $C_{20}H_{17}F_3N_4O_3$ (%): C 57.42, H 4.10, N 13.39; Found: C 57.33, H 3.98, N 13.01.

6.4.6. 1-(4-(4,6-Dimethylpyrimidin-2-yloxy)phenyl)-3-(4-fluorophenyl)urea (**4d**)

White solid, yield: 85%, mp 219.6–220.4 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.32 (s, 6H, CH_3), 6.99(s, 1H, CH), 7.07 (d, 2H, J = 9.0 Hz, ArH), 7.12 (d, 2H, J = 9.0 Hz, ArH), 7.45–7.50 (m, 4H, ArH), 8.69 (s, 1H, NH), 8.72 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 115.3, 119.4, 120.0, 121.8, 136.1, 136.5, 147.5, 152.7, 156.6, 158.1, 164.7, 169.4; Anal. Calcd. for $C_{19}H_{17}FN_4O_2$ (%): C 64.76, H 4.86, N 15.90; Found: C 64.38, H 4.60, N 15.60.

6.4.7. 1-(4-chlorophenyl)-3-(4-(4,6-dimethylpyrimidin-2-yloxy)phenyl)urea (**4e**)

White solid, yield: 83%, mp 225.8–226.7 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.32 (s, 6H, CH_3), 6.99 (s, 1H, CH), 7.07 (d, 2H, J = 8.4 Hz, ArH), 7.33 (d, 2H, J = 9.0 Hz, ArH), 7.45–7.50 (m, 4H, ArH), 8.73 (s, 1H, NH), 8.83 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 115.3, 119.4, 119.6, 122.0, 125.3, 128.6, 136.3, 138.8, 147.6, 152.5, 164.6, 169.4; Anal. Calcd. for $C_{19}H_{17}ClN_4O_2$ (%): C 61.87, H 4.65, N 15.19; Found: C 62.08, H 4.37, N 15.06.

6.4.8. 1-(2,4-dichlorophenyl)-3-(4-(4,6-dimethylpyrimidin-2-yloxy)phenyl)urea (**4f**)

White solid, yield: 83%, mp 220.3–222.1 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.32 (s, 6H, CH_3), 6.70 (s, 1H, CH), 7.09 (d, 2H, J = 9.0 Hz, ArH), 7.40–7.48 (m, 3H, ArH), 7.63 (s,1H, ArH), 8.21 (d, 2H, J = 9.0 Hz, ArH), 8.40 (s, 1H, NH), 9.48 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 115.3, 119.4, 122.0, 122.5, 126.0, 127.6, 127.7, 128.5, 135.3, 136.1, 147.8, 152.0, 164.6, 169.4; Anal. Calcd. for $C_{19}H_{16}Cl_2N_4O_2$ (%): C 56.59, H 4.00, N 13.89; Found: C 56.77, H 3.84, N 14.28.

6.4.9. 1-(4-(4,6-Dimethylpyrimidin-2-ylthio)phenyl)-3-phenylurea (**4g**)

White solid, yield: 86%, mp 211.4–213.2 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.26 (s, 6H, CH_3), 6.94 (s, 1H, CH), 6.97–6.99 (m, 1H, ArH), 7.26–7.29 (m, 2H, ArH), 7.44–7.52 (m,6H, ArH), 8.73 (s, 1H, NH), 8.87 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 116.4, 118.2, 118.4, 121.0, 122.0, 128.8, 135.9, 139.4, 140.7, 152.3, 167.2, 170.5; Anal. Calcd. for $C_{19}H_{18}N_4OS$ (%): C 65.12, H 5.18, N 15.99; Found: C 65.39, H 4.96, N 15.98.

6.4.10. 1-(4-(4,6-Dimethylpyrimidin-2-ylthio)phenyl)-3-m-tolylurea (**4h**)

White solid, yield: 80%, mp 196.1–197.8 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.25 (s, 9H, CH_3), 6.78 (s, 1H, ArH), 6.93 (s, 1H, CH), 7.12–7.28 (m, 3H, ArH), 7.43 (d, 2H, J = 7.8 Hz, ArH), 7.50 (d, 2H, J = 8.4 Hz, ArH), 8.63 (s, 1H, NH), 8.83 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 21.2, 23.4, 115.6, 116.4, 118.4, 118.8, 121.0, 122.7, 128.7, 135.9, 138.0, 139.3, 140.7, 152.3, 167.2, 170.5; Anal. Calcd. for

$C_{20}H_{20}N_4OS$ (%): C 65.91, H 5.53, N 15.37; Found: C 65.64, H 5.15, N 15.59.

*6.4.11. 1-(4-(4,6-Dimethylpyrimidin-2-ylthio)phenyl)-3-(4-(trifluoromethoxy)phenyl) urea (**4i**)*

White solid, yield: 82%, mp 218.8–219.5 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.25 (s, 6H, CH_3), 6.93 (s, 1H, CH), 7.27 (d, 2H, J = 9.0 Hz, ArH), 7.44 (d, 2H, J = 8.4 Hz, ArH), 7.50–7.55 (m, 4H, ArH), 8.91 (s, 1H, NH), 8.93 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.3, 116.4, 118.7, 119.4, 119.5, 121.4, 121.8, 135.9, 138.9, 140.5, 142.8, 152.3, 167.1, 170.6; Anal. Calcd. for $C_{20}H_{17}F_3N_4O_2S$ (%): C 55.29, H 3.94, N 12.90; Found: C 55.66, H 4.14, N 12.76.

*6.4.12. 1-(4-(4,6-Dimethylpyrimidin-2-ylthio)phenyl)-3-(4-fluorophenyl)urea (**4j**)*

White solid, yield: 84%, mp 207.9–210.0 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.28 (s, 6H, CH_3), 6.96 (s, 1H, CH), 7.13 (d, 2H, J = 8.4 Hz, ArH), 7.45–7.48 (m, 4H, ArH), 7.53 (d, 2H, J = 8.4 Hz, ArH), 8.79 (s, 1H, NH), 8.89 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.4, 115.3, 116.4, 118.5, 120.1, 121.1, 135.9, 140.7, 152.5, 156.7, 158.3, 167.1, 170.6; Anal. Calcd. for $C_{19}H_{17}FN_4OS$ (%): C 61.94, H 4.65, N 15.21; Found: C 62.21, H 4.32, N 15.18.

*6.4.13. 1-(4-chlorophenyl)-3-(4-(4,6-dimethylpyrimidin-2-ylthio) phenyl)urea (**4k**)*

White solid, yield: 81%, mp 228.3–229.4 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.28 (s, 6H, CH_3), 6.96 (s, 1H, CH), 7.33–7.54 (m, 8H, ArH), 8.90 (s, 1H, NH), 8.93 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.4, 116.4, 118.6, 119.7, 121.3, 125.6, 128.6, 135.9, 138.6, 140.5, 152.3, 167.1, 170.5; Anal. Calcd. for $C_{19}H_{17}ClN_4OS$ (%): C 59.29, H 4.45, N 14.56; Found: C 59.60, H 4.46, N 14.34.

*6.4.14. 1-(2,4-dichlorophenyl)-3-(4-(4,6-dimethylpyrimidin-2-ylthio)phenyl)urea (**4l**)*

White solid, yield: 86%, mp 246.8–248.5 °C; ^{1}H NMR (DMSO-d_6, 600 MHz): 2.28 (s, 6H, CH_3), 6.97 (s, 1H, CH), 7.40–7.55 (m, 6H, ArH), 7.65 (s, 1H, ArH), 8.20 (s, 1H, NH), 8.47 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): 23.4, 116.4, 118.6, 121.6, 122.1, 122.7, 126.3, 127.6, 128.5, 135.1, 136.0, 140.2, 151.9, 167.1, 170.5; Anal. Calcd. for $C_{19}H_{16}Cl_2N_4OS$ (%): C 54.42, H 3.85, N 13.36; Found: C 54.52, H 3.58, N 13.27.

6.5. Pharmacology

The in vitro cytotoxicity of the synthesized compounds against different cancer cell lines was performed with the MTT assay according to the Mosmann's method [15]. The MTT assay is based on the reduction of the soluble 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into a blue–purple formazan product, mainly by mitochondrial reductase activity inside living cells. The cells used in cytotoxicity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2Y′ 104/mL) were plated in 96-well culture plates and incubated at 37 °C in a 5% CO_2 incubator. After 12 h, the test sample (2 mL) was added to the cells (2Y′ 104) in 96-well plates and cultured at 37 °C for 3 days. The cultured cells were mixed with 20 mL of MTT solution and incubated for 4 h at 37 °C. The supernatant was carefully removed from each well and 100 mL of DMSO was added to each well to dissolve the formazan crystals which were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the absorbance of each well was measured by a microplate reader using a test wavelength of 570 nm. The results were expressed as the IC_{50}, which is the concentration of the drugs inducing a 50% inhibition of cell growth of treated cells when compared to the growth of control cells. Each experiment was performed at least 3 times. There was a good reproducibility between replicate wells with standard errors below 10%.

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