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Research Article

Synthesis of Substituted Thieno[2,3-*d*]pyrimidin-4-ones and Their Testing for Evaluation of Cytotoxic Activity on Mammalian Cell Models

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From 2-amino-3-ethoxycarbonyl-4,5-dimethyl-, -polymethylenethiophenes (**1-4**) were synthesized 2,3-disubstituted thieno[2,3-d]dihydropyrrolo-, tetrahydropyrido-, and tetrahydroazepino[1,2-a]pyrimidin-4-ones (**5-16**) for pharmacological investigations. The 12 compounds (**5-16**) were individually evaluated for their antiproliferative activities on mammalian cancer cell models. All tested compounds showed weak affection on human cervix adenocarcinoma cells (HeLa) whereas some of the tested compounds exhibited more consistent inhibition of cell growth on murine myeloma cells (P3X). In both cases some compounds enhanced cell proliferation.

1. Introduction

Thieno[2,3-*d*]pyrimidin-4-ones are a large group of heterocyclic compounds [1] and some of them show antiviral [2], antimicrobial [3–10], and antibacterial properties [11]. Fused tri- and tetracyclic thieno[2,3-d]pyrimidin-4-ones are synthesized by many methods and among them some compounds have fungicidal, antibacterial, and antiinflammatory activities [12–19], and their substituted derivatives were reported as 17β-HSD1 inhibitors [20].

These findings clearly show the potential importance of such molecules as active principles of new pharmaceuticals and therefore the development of effective methods of synthesis and searching of biological activities among new synthesized compounds are a very important direction. Prompted by the various biological activities of thieno[2,3-*d*]pyrimidin-4-ones and its substituted derivatives, we envisioned our approach towards the synthesis of a novel series of thieno[2,3-*d*]pyrimidin-4-ones derivatives and to evaluate their possible cytotoxic activity on mammalian cell models.

2. Materials and Methods

2.1. Chemicals and Reagents. ^{1}H NMR spectra were recorded on Unity 400$^+$ (400 MHz) in $CDCl_3$. Chemical shifts of ^{1}H were measured relative to HMDS as internal standard. IR spectra were recorded as KBr pellets on an IR Fury System 2000 (Perkin-Elmer). Melting points were measured on a Boetius (Germany) and MEL-TEMP (USA) apparatus and are not uncorrected. Reactions were monitored by TLC on Sorbfil (Russia) and Whatman UV-254 (Germany) (eluent: benzene-methanol, 2 : 1 (A) and benzene-methanol, 5 : 1 (B)), which were visualized under UV radiation.

Cell culture media, supplements, and dimethyl sulfoxide (DMSO) were purchased from Roth and Greiner

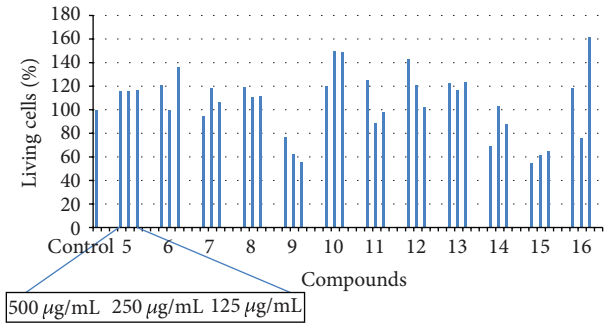


Figure 1: Cytotoxic action of compounds **5–16** on HeLa cells after 24-hour culturing (MTT test).

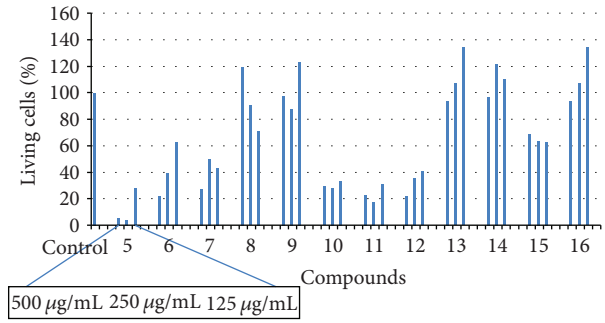


Figure 2: Cytotoxic action of compounds **5–16** on P3X cells after 24-hour culturing (MTT test).

Labortechnik (Italy). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma and Gibco (Invitrogen, Italy).

2.1.1. Cytotoxic Activity. The evaluation of cytotoxic activity of the tested samples was carried out on HeLa (human epithelial cervical cancer, *ATCC* CCL-2) and P3X (murine myeloma, *ATCC* CRL-1580) cell models. For this purpose cells were cultured at 37°C, 90% of humidity and 5% CO_2 in 96-well plates (5×10^4 cells/well for HeLa and 1×10^5 for P3X cells) in DMEM medium culture (pH 7.4), containing 10% fetal bovine serum and 50 units/mL penicillin/streptomycin. Cells were treated for 24 hours with the individual substance (in concentrations of 500, 250, and 62.5 μg/mL, resp.). Controls consisted of untreated cancer cells incubated for 24 hours. The obtained results were recalculated in percentage with respect to the control. All experiments were conducted in 3-time frequency.

2.1.2. MTT Assays. Cytotoxic activity was evaluated by using the MTT test based on reducing [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) with the participation of cytoplasmatic dehydrogenases in insoluble formazan, the quantity of which correlates with metabolic activity of cells and a level of cell proliferation [21]. The medium was replaced 4 hrs before the incubation period (100 μL DMEM), containing 0.5 mg/mL MTT, and incubated for 3 hours at 37°C. At the end of the experiment the formed crystals of formazan were dissolved in DMSO and the absorbance was determined by a plate lecturer. Cytotoxic activity of substances was determined by percent of discolor in control samples at 595 nm. Cytotoxic activity is determined by the change in absorbance relative to the controls and has been expressed as cell viability rate (%).

3. Experimental Part

3.1. Sample Preparation. Compounds **1–4** were synthesized following to the procedure of Gewald et al., as already reported [17] with some minor modifications. To a suspension containing of 0.4 mole ketone, 0.4 mole ethyl cyanoacetate, and 0.44 g sulphur powder in 120 mL ethanol, 40 mL diethylamine was added dropwise and the temperature of the reaction mixture was kept at 45–50°C for 3 h; the reaction solution was allowed to stand overnight at +5°C (in Gewalds procedure [17], a reactionary mixture leave in a refrigerator at some times). 700 mL Distilled water was added and was mixed for 3 h at room temperature. The formed crystals were filtered off and washed with water and dried. The products **1–4** were purified by recrystallization from corresponding solvent. Data on the prepared compounds **1–4** are given in Table 1.

Compounds **5–16** were obtained by modified method, which were reported previously [19, 22]. 2-Amino-3-ethoxycarbonyl-4,5-dimethyl- (**1**), -4,5-tri-, -tetra-, -pentamethylene thiophenes (**2–4**) were synthesized by some minor modifications of the already mentioned methodology [17].

2,3-Disubstituted thieno[2,3-d]dihydropyrrolo-, tetrahydropyrido-, and tetrahydroazepino [1,2-a]pyrimidin-4-ones. were synthesized by modified method [22]. *(General Procedure).* To a mixture containing of 0.2 mole of compounds **1–4** and 0.3 mole lactam, 0.72 mole phosphorusoxychloride was added dropwise (0.5 h) at 0/+2°C. The reaction mixture was kept at 96–98°C for 2 h and subsequently allowed to stand overnight at room temperature; grinded ice was then added for decomposition of a reactionary mixture and 12% NH_4OH was added up to pH = 9-10. The formed crystals were filtered off and washed with water and dried. The products **5–16** were purified by recrystallization from corresponding solvent (see Table 1). Data on the prepared compounds **5–16** are given in Table 1. The structure of the synthesized compounds was elucidated by their ^{1}H NMR and IR spectral data analysis.

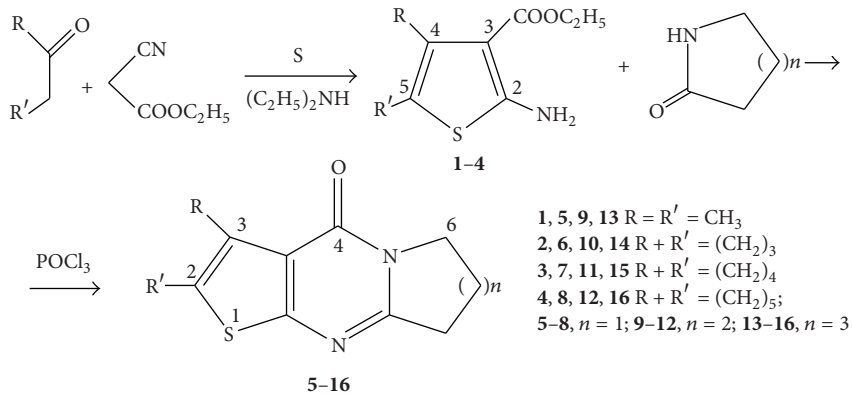
4. Results and Discussion

2,3-Disubstituted thieno[2,3-*d*]dihydropyrrolo-, tetrahydropyrido-, and tetrahydroazepino[1,2-*a*]pyrimidin-4-ones, **5–16**, are synthesized from 2-amino-3-ethoxycarbonyl-4,5-dimethyl-, -polymethylene thiophenes (compounds **1–4**) by condensation reaction with lactams [12, 19, 22]. The thiophene analogues (**1–4**) may be synthesized from aliphatic or cyclic ketones (Scheme 1 and Table 1) [17].

Synthesis of compounds **5–16** was already has been carried out by the method changed by us [19, 22]. Addition to reagents of $POCl_3$ at cooling (0°C), instead of at room

Table 1: Some physical-chemical parameters of synthesized compounds **(1–16)**.

Compound no.	Yield, % (%, [Lit.])	Mp, °C (solvent) (Mp, [Lit.])	R_f (Eluent)	Empiric formula
1	86 (42, [17])	90–92 (cyclohexane) (88–90, [17])	0.70 (A)	$C_9H_{13}NO_2S$
2	81 (45, [17])	87–89 (hexane) (87–89, [17])	0.65 (A)	$C_{10}H_{13}NO_2S$
3	85 (82, [17])	98–100 (hexane) (96–98, [17])	0.43 (B)	$C_{11}H_{15}NO_2S$
4	82	70-71 (hexane) (68–70, [17])	0.74 (B)	$C_{12}H_{17}NO_2S$
5	86	144-145 (hexane) (144-145, [17])	0.70 (A)	$C_{11}H_{12}N_2OS$
6	90 (54, [14])	202-203 (methanol) (200-201, [14])	0.65 (A)	$C_{12}H_{12}N_2OS$
7	92 (60, [14])	214-215 (ethanol) (212–214, [14])	0.43 (B)	$C_{13}H_{14}N_2OS$
8	92 (62, [14])	157-158 (ethanol) (156–158, [14])	0.74 (B)	$C_{14}H_{16}N_2OS$
9	85	115–117 (heptane) (115–117, [14])	0.75 (B)	$C_{12}H_{14}N_2OS$
10	82 (80, [14])	182-183 (heptane) (180–183, [14])	0.75 (B)	$C_{13}H_{14}N_2OS$
11	88 (85, [14])	218–220 (ethanol) (215-216, [14])	0.76 (B)	$C_{14}H_{16}N_2OS$
12	92 (78, [14])	156–158 (ethanol) (155–157, [14])	0.80 (B)	$C_{15}H_{18}N_2OS$
13	88	159–161 (heptane) (158–160, [15])	0.82 (B)	$C_{13}H_{16}N_2OS$
14	85 (75, [15])	197-198 (heptane) (196–198, [14])	0.80 (B)	$C_{14}H_{16}N_2OS$
15	91 (67, [14])	155–157 (ethanol) (150–152, [14])	0.81 (B)	$C_{15}H_{18}N_2OS$
16	90 (65, [14])	161–163 (heptane) (159-160, [14])	0.86 (B)	$C_{16}H_{20}N_2OS$



Scheme 1: Synthesis of thieno [2,3-*d*]pyrimidin-4-One derivatives.

temperature (20°C), the increasing in duration of reaction and processing of a reaction mixture by ice water (thus decomposition of reaction products decreases) have allowed to obtain main products **5–16** in high yields (82–96%) (Table 1).

Our results showed that the increase of methylene groups, that is, at transition from dihydropyrrolo- (**5**), tetrahydropyrido- (**9**) and tetrahydroazepino- (**13**) derivatives, the inhibition activity on murine myeloma cells (P3X) of compounds in the line **5** > **9** > **13** (250 μg/mL)

Table 2: IC50 determination of substances **5–16** on HeLa and P3X cells after 24-hour culturing (MTT assay).

Sample	P3X	Hela
5	86,4 μg/mL	>500 μg/mL
6	195 μg/mL	>500 μg/mL
7	249,7 μg/mL	>500 μg/mL
8	>500 μg/mL	>500 μg/mL
9	>500 μg/mL	>500 μg/mL
10	93,1 μg/mL	>500 μg/mL
11	90,0 μg/mL	>500 μg/mL
12	103,8 μg/mL	>500 μg/mL
13	>500 μg/mL	>500 μg/mL
14	>500 μg/mL	>500 μg/mL
15	>500 μg/mL	>500 μg/mL
16	>500 μg/mL	>500 μg/mL

decreased. The same behavior was observed for 5,6-dimethyl- (**5**), -trimethylene- (**6**), -tetramethylene- (**7**), and -pentamethylene- (**8**) derivatives in the line of compounds **5** > **6** > **7** > **8**. Among of them, compound **5** resulted as the most active and in this view, further investigations with derivatives of 2,3-dimethylthieno [2,3-*d*]dihydropyrrolo [1,2-*a*]pyrimidin-4-one (**5**) will be carried out in order to evaluate both the affecting level of single derivatives and their cellular targets. The **5–16** obtained compounds exhibited moderate cytotoxic activity on HeLa cells.

4.1. NMR Studies of Compounds **5–16**. The structure of the synthesized compounds is confirmed with reference of protons H-6 (in the field of 4.1 ppm) and H-8 (in the field of 3.1 ppm) and comparison of the received spectra with spectra of known related compounds [12]. So, in spectrum ^{1}H NMR chemical shift of protons methylene groups (H-6) is resounded rather in the weaker field (4.09–4.11 ppm) as a two-proton triplet; 8-CH_2 group is shown in the field of 3.07–3.10 ppm (triplet), and 7-CH_2 groups - at 2.18–2.25 ppm as two-proton multiplets. Methylene groups of 3-CH_2 have chemical shift for compounds **6–8** in an interval 2.94–3.27 ppm (triplet), 2-CH_2- at 2.69–2.87 ppm, 2-CH_2–$\underline{CH_2}$ (compound **6**) and 3-CH_2–$\underline{CH_2}$ (compound **8**) - in the field of 2.35–2.41 ppm and at 1.82 ppm (both as multiplets); four-proton multiplets 2-CH_2–($\underline{CH_2}$)$_2$ compounds **7,8** are shown in rather stronger field (1.75–1.81 ppm and 1.59–1.62 ppm, accordingly). Protons of methyl groups (**5**) -2-CH_3 and 3-CH_3 have chemical shift at 2.30 ppm and 2.41 ppm (both as singlet).

*2,3-Dimethylthieno [2,3-d]dihydropyrrolo]1,2-a]pyrimidin-4-one (**5**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 2.18–2.24 (2H, m, H-7); 2.30 (3H, s, 2-CH_3); 2.41 (3H, s, 3-CH_3); 3.07 (2H, t, J = 8.1, H-8); 4.10 (2H, t, J = 7.1, H-6);

IR (KBr), ν (cm^{-1}): 2926 (ν_{CH3}), 1664 ($\nu_{C=O}$), 1542 ($\nu_{C=N}$), 1463 (ν_{C-N}).

*2,3-Trimethylenethieno [2,3-d]dihydropyrrolo]1,2-a]pyrimidin-4-one (**6**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 2.19–2.25 (2H, m, H-7); 2.35–2.41 (2H, m, 2-$CH_2\underline{CH_2}$); 2.87 (2H, t, J = 7.1, 2-CH_2); 3.0 (2H, t, J = 7.1, 3-CH_2); 3.10 (2H, t, J = 8.0, H-8); 4.11 (2H, t, J = 7.2, H-6);

IR (KBr), ν (cm^{-1}): 2957 (ν_{CH2}), 1668 ($\nu_{C=O}$), 1577 ($\nu_{C=N}$), 1463 (ν_{C-N}).

*2,3-Tetramethylenethieno [2,3-d]dihydropyrrolo]1,2-a]pyrimidin-4-one (**7**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.75–1.81 (4H, m, 2-CH_2($\underline{CH_2}$)$_2$); 2.19–2.24 (2H, m, H-7); 2.69 (2H, t, J = 5.8, 2-CH_2); 2.94 (2H, t, J = 6.2, 3-CH_2); 3.07 (2H, t, J = 7.9, H-8); 4.09 (2H, t, J = 7.3, H-6);

IR (KBr), ν (cm^{-1}): 2980 (ν_{CH2}), 1662 ($\nu_{C=O}$), 1584 ($\nu_{C=N}$), 1475 (ν_{C-N}).

*2,3-Pentamethylenethieno [2,3-d]dihydropyrrolo]1,2-a]pyrimidin-4-one (**8**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.59–1.62 (4H, m, 2-CH_2($\underline{CH_2}$)$_2$); 1.82 (2H, m, 3-$CH_2\underline{CH_2}$); 2.18–2.24 (2H, m, H-7); 2.76 (2H, t, J = 5.7, 2-CH_2); 3.07 (2H, t, J = 7.9, H-8); 3.27 (2H, t, J = 5.7, 3-CH_2); 4.10 (2H, t, J = 7.3, H-6);

IR (KBr), ν (cm^{-1}): 2930 (ν_{CH2}), 1660 ($\nu_{C=O}$), 1548 ($\nu_{C=N}$), 1469 (ν_{C-N}).

*2,3-Dimethylthieno [2,3-d]tetrahydropyrido]1,2-a]pyrimidin-4-one (**9**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.83–1.92 (4H, m, H-7,8); 2.30 (3H, s, 2-CH_3); 2.40 (3H, s, 3-CH_3); 2.88 (2H, t, J = 6.7, H-9); 3.95 (2H, t, J = 6.2, H-6);

IR (KBr), ν (cm^{-1}): 2945 (ν_{CH3}), 1665 ($\nu_{C=O}$), 1539 ($\nu_{C=N}$), 1446 (ν_{C-N}).

*2,3-Trimethylenethieno [2,3-d]tetrahydropyrido]1,2-a]pyrimidin-4-one (**10**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.84–1.89 (2H, m, 2-$CH_2\underline{CH_2}$); 1.90–1.94 (2H, m, H-8); 2.37–2.40 (2H, m, H-7); 2.87 (2H, t, J = 7.5, 2-CH_2); 2.90 (2H, t, J = 6.8, 3-CH_2); 3.0 (2H, t, J = 7.3, H-9); 3.98 (2H, t, J = 6.0, H-6);

IR (KBr), ν (cm^{-1}): 2952 (ν_{CH2}), 1669 ($\nu_{C=O}$), 1530 ($\nu_{C=N}$), 1487 (ν_{C-N}).

*2,3-Tetramethylenethieno [2,3-d]tetrahydropyrido]1,2-a]pyrimidin-4-one (**11**)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.75–1.83 (4H, m, 2-CH_2($\underline{CH_2}$)$_2$); 1.85–1.94 (4H, m, H-7,8); 2.69 (2H, t, J = 6.0, 2-CH_2); 2.91 (2H, t, J = 6.7, 3-CH_2); 2.95 (2H, t, J = 5.9, H-9); 3.96 (2H, t, J = 6.3, H-6);

IR (KBr), ν (cm^{-1}): 2932 (ν_{CH2}), 1665 ($\nu_{C=O}$), 1539 ($\nu_{C=N}$), 1455 (ν_{C-N}).

*2,3-Pentamethylenethieno [2,3-d]tetrahydropyrido]1,2-a]pyrimidin-4-one (***12***)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.57–1.66 (4H, m, 2-$CH_2(C\underline{H}_2)_2$); 1.81–1.86 (4H, m, H-7,8); 1.89–1.93 (2H, m, 3-$CH_2C\underline{H}_2$); 2.75 (2H, t, J = 5.6, 2-CH_2); 2.88 (2H, t, J = 6.7, 3-CH_2); 3.27 (2H, t, J = 5.7, H-9); 3.95 (2H, t, J = 6.2, H-6);

IR (KBr), ν (cm^{-1}): 2917 (ν_{CH2}), 1662 ($\nu_{C=O}$), 1543 ($\nu_{C=N}$), 1487 (ν_{C-N}).

*2,3-Dimethylthieno [2,3-d]tetrahydroazepino]1,2-a]pyrimidin-4-one (***13***)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.71–1.79 (6H, m, H-7,8,9); 2.30 (3H, s, 2-CH_3); 2.40 (3H, s, 3-CH_3); 2.95 (2H, t, J = 5.0, H-10); 4.29 (2H, t, J = 4.9, H-6);

IR (KBr), ν (cm^{-1}): 2935 (ν_{CH2}), 1671 ($\nu_{C=O}$), 1546 ($\nu_{C=N}$), 1492 (ν_{C-N}).

*2,3-Trimethylenethieno [2,3-d]tetrahydroazepino]1,2-a]pyrimidin-4-one (***14***)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.71–1.79 (6H, m, H-7,8,9); 2.37–2.42 (2H, m, 2-$CH_2C\underline{H}_2$); 2.87 (2H, t, J = 5.6, 2-CH_2); 2.97 (2H, t, J = 5.4, 3-CH_2); 3.0 (2H, t, J = 5.6, H-10); 4.31 (2H, t, J = 4.9, H-6).

*2,3-Tetramethylenethieno [2,3-d]tetrahydroazepino]1,2-a]pyrimidin-4-one (***15***)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.70–1.82 (10H, m, 2-$CH_2(C\underline{H}_2)_2$, H-7,8,9); 2.69 (2H, t, J = 5.9, 2-CH_2); 2.93 (2H, t, J = 5.9, 3-CH_2); 2.95 (2H, t, J = 6.7, H-10); 4.28 (2H, t, J = 4.9, H-6).

*2,3-Pentamethylenethieno [2,3-d]tetrahydroazepino]1,2-a]pyrimidin-4-one (***16***)*

^{1}H NMR ($CDCl_3$), δ, J (Hz): 1.57–1.85 (12H, m, 2-$CH_2(C\underline{H}_2)_3$, H-7,8,9); 2.75 (2H, t, J = 5.4, 2-CH_2); 2.95 (2H, t, J = 5.2, 3-CH_2); 3.27 (2H, t, J = 5.8, H-10); 4.29 (2H, t, J = 4.9, H-6).

4.2. Cytotoxic Activity. We investigated the synthesized compounds for their cytotoxic activity on mammalian cancer cell models such as HeLa (human epithelial cervical cancer) and P3X (murine myeloma) cells. HeLa cells were seeded into 96-well plates at a concentration of 5.0×10^4 cells/mL and P3X cells were seeded into 96-well plates at a concentration of 1×10^5 cells/mL. After 24 hours, cells were treated with solubilized synthesized compounds **5–16**, which were diluted with culture medium to the final concentrations of, respectively, 500, 250, and 125 μg/mL. After 24 hours cell viability was determined by MTT assay.

The results of the cytotoxicity tests are shown in Table 2 and the graphics are reported in Figure 1 for HeLa cells and Figure 2 for P3X cells. All the tested substances showed weak antiproliferative activities against HeLa cells and as shown by our experiments many compounds enhance proliferation of such cancer cell model. These compounds exhibited varying degrees of cytotoxic activity against P3X cell lines when tested of P3X murine myeloma cells. The substance **5** exhibited potent antiproliferative activity with IC50 values of 86,4 μg/mL; substances **6**, **7**, **10**, **11**, **12** showed a moderate cytotoxic activity in P3X cells growth and the level of affection seemed dependent on the administered concentration. Substances **8**, **9**, **13–16** demonstrated weak inhibition of P3X cell proliferation, with IC50 values higher than 500 μg/mL. However some compounds showed contrasting results by enhancing the proliferation of both the cell lines tested.

5. Conclusion

We synthesized a series of thieno[2,3-*d*]pyrimidin-4-ones in high yields; the synthesized **1–4** compounds were used for the synthesis of **5–16** compounds. The advantages of the obtained **5–16** compounds are low cost of the starting chemicals and simple experimental procedure of synthesis. The **5–16** obtained compounds exhibited moderate cytotoxic activity on HeLa cells, whereas more consistent antiproliferative activity was detected in P3X myeloma murine cells and in this view further studies will be addressed to investigate the cellular targets of compounds showing antiproliferative activity. In both cell models some of the tested compounds showed to enhance cell proliferation.

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