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Synthesis and anticancer activity of acyl thioureas bearing pyrazole moiety

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ABSTRACT

In this work novel organic based compounds, acyl thiourea derivatives were synthesized and their anticancer activities were investigated. A new series of acyl thiourea derivatives containing pyrazole ring were prepared in good yield through one pot reaction of 4-benzoyl-1, 5-diphenyl-1*H*-pyrazole-3-carbonyl chloride with ammonium thiocyanate and various amines. The structures of the newly synthesized compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. Anticancer activities of synthesized compounds were evaluated on human colon, liver and leukemia cancer cell lines. Cell culture studies have demonstrated significant toxicity of the compounds on the cell lines, and the levels of toxicity have altered in the presence of various side groups. These results confirm that novel pyrazolyl acyl thioureas derived compounds may be utilized for cancer treatment. Furthermore, these compounds have a great potential and significance for further investigations.

1. Introduction

Today, cancer is one of the biggest health problems in the world. Especially, the number of deaths from leukemia, colon, and liver cancer is increasing gradually worldwide. Pharmaceutical industries spend billions of dollars for the development of effective agents in the diagnosis and treatment of cancer each year. Therefore, many organic based cytotoxic agents have been discovered, and they are extensively applied for treatment of cancer.[1,2]

Acyl thiourea derivatives have been increasingly important with a wide diversity of applications in heterocyclic chemistry, metal complexes, molecular electronics and exhibit an array of biological activities.[3–7] Some of them are employed as fungicidal, antiviral, antimicrobial,[8] parasiticidal,[9] antitumoral[10] and pesticidal agent.[11–13] Acyl thiourea compounds cambinol and tenovin-1 are small molecule inhibitors of the NAD^{+}-dependent family of protein deacetylases known as the sirtuins (Fig. 1). There is considerable interest in inhibitors of this enzyme family due to possible applications in both cancer and neurodegenerative disease therapy.[14–17] 2-(benzoylcarbamothioylamino)-5,5-dimethyl-4,7-dihydrothieno [2,3-*c*] pyran-3-carboxylic acid (PubChem CID 1067700) has been reported as an inhibitor of nucleotide binding by Ras-related GTPases (Fig. 1).[18] A new series of acylthiourea analogs inhibited microsomal epoxide hydrolase (mEH) is a liver enzyme.[19] In recent years, You et al. reported that some acylthiourea compounds showed HIV inhibition activity.[20]

The chemistry of pyrazole derivatives have been the subject of medical research due to the various biological and pharmacological properties such as antitumor,[21] antimicrobial,[22,23] anti-inflammatory,[24] antiviral,[25] antifungal,[26] anticonvulsant,[27] analgesic agents.[28] Anticancer effects of pyrazole was first discovered half a century ago with its high toxicity even at 0.15 mmol/kg/day dose.[29,30] In order to overcome this effect, 1-carboxamidopyrazole and 1-thiocarbamoylpyrazole were synthesized. They showed significant anticancer effects on animal experiments, but failed to pass the clinical evaluation.[31,32] In order to find better antitumor agent, a large series of pyrazole derivatives were synthesized and tested over the years. So the use of this powerful pharmacophore is still very popular.[33–35]

In the present study, we synthesized novel pyrazolyl acyl thioureas by the addition of various aromatic amines to 4-benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carbonyl isothiocyanate. The structures of the synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis studies. Anticancer potential of compounds was examined in human leukemia, colon, and liver cancer cell lines. Our compounds may offer new agents for treatment of leukemia, colon and liver cancer.

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2. Results and discussion

2.1. Chemistry

In the present work, one pot synthesis of pyrazolyl acyl thioureas were accomplished as described in Scheme 1. The starting material 4-benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carbonyl chloride (**1**) were prepared as described earlier.[36]

The synthesis began by treating **1** with ammonium thiocyanate. The resulting acyl isothiocyanate was not isolated from the mixture and was converted into the corresponding acyl thiourea (**3**) by adding various amines. The reaction was carried out under reflux condition. All crude products can be purified by column chromatography. The target compounds were obtained in 62–79% yields (Table 1). All the structures of newly synthesized compounds were elucidated on the basis of their elemental analysis and spectroscopic data.

The IR spectra of **3a** showed absorption band at 3368 cm^{-1} due to the presence of the NH group. The absorptions of C=O and C=S groups were observed at 1682, 1665 and 1176 cm^{-1} respectively. The medium strong C=O band in the IR spectra of all the compounds appeared at 1720–1620 cm^{-1}, which is lower than that of the ordinary carbonyl absorption (1730–1650 cm^{-1}). The formation of H-bond leads an increase of their polarity, so the strength of their double bond decreased, and absorption moved to lower wave number.[37] The ^{1}H NMR spectra of **3a** exhibited two broad signals at 12.06 and 10.02 ppm, which were assigned the two N–H protons. The signals of aromatic H-atoms were observed as multiplets between 7.88 and 7.16 ppm. ^{13}C NMR for **3a** showed peaks at δ 190.99, 159.97 ppm for C=O and 178.07 ppm for C=S. C=C and C=N signals arised at 144.52–123.10 ppm. ^{13}C NMR spectra of **3b**–**3k** were similar to those of **3a**, except for the signals of the substituent at phenyl ring, which exhibited characteristic resonances with appropriate chemical shifts.

2.2. Anticancer activity of new pyrazole derivatives

In this study, the anticancer activity of the 11 synthesized pyrazolyl acyl thiourea derivatives has been evaluated on human cancer cell lines, representing liver, leukemia and colon cancer (Table 2). Cells were exposed to five concentrations ranging from 10^{-4} to 10^{-8} M compounds. After 24 and 48 h, a XTT test protocol was applied, and the results were compared with the control cells (untreated cells).[38,39] In order to determine the inhibitory effects of compounds, the cell viability of control has been accepted as 100%, and the other results were proportioned according to the control value.

According to the results of cell culture studies, all compounds showed antitumor activity at the two extreme concentrations (10^{-4} and 10^{-5} M). Moreover, degree of the compound inhibition level is proportional to the incubation time for 24–48 h time frame. The results of these two concentrations were statistically significant.

The tested compound **3a, 3b**, **3d**, **3h**, **3j** and **3k** showed significant inhibition over 50% on the Jurkat cells at 24 h and 48 h (Fig. 2). However, compound **3a**, **3d** and **3f** showed anti-tumor activity at 24 h. Moreover, the results of compound **3c** and **3e** are not significant statistically. All of the compounds but **3i** showed significant anti-cancer activity at 24 h and 48 h in DLD-1 cells (Fig. 3) as HepG2 cells. Overall, compounds inhibit HepG2 cells better than DLD-1 and inhibit DLD-1 cells better than Jurkat cells since compounds **3c**, **3e**, **3g**, **3i**, and **3k** have weak anticancer activity (Fig. 2). Compound **3i** has the lowest anticancer activity among the compounds in this study. **3i** compound cannot adequately inhibit DLD-1 cancer cells and ninety five percent of the cells survive in the presence of this compound (Fig. 3). Similarly, this set of compounds share significant anticancer activity in HepG2 cells (Fig. 4).

Side group of the compounds is one of the most important factors in the emergence of anticancer effect. Generally, the addition of phenyl groups is excessively increased the anticancer effect of compounds.[30] Therefore, all of the compounds have greatly shown anticancer activity for colon, leukemia and liver cancer cells. Compounds **3e**, **3f** and **3g** have different halogenic side groups (F^-, Cl^- and Br^- respectively), but they display approximately similar anticancer profiles. Compound **3a** (phenyl group with no halogenic side chain) has higher anticancer activity than compound **3e**, **3f,** and **3g** during 24 hours in Jurkat cell line. Out of **3e**, **3f**, and **3g**, **3f** displays similar activity to **3a**. The electronegativity of F (**3e**) and bulky group of Br (**3g**) as side chain most likely perturb the anticancer activity of phenyl compound (**3a**). The Cl side group of **3f** presumably shows optimal positioning and do not perturb the activity of its phenylic body. Side chain length may enhance this optimal positioning as revealed by **3h**. Extra ring on phenylic compound (**3h**) which cause steric hindrance may or may not facilitate optimal positioning. **3h** compound increases anticancer activity in Jurkat cells while the same compound decreases anticancer

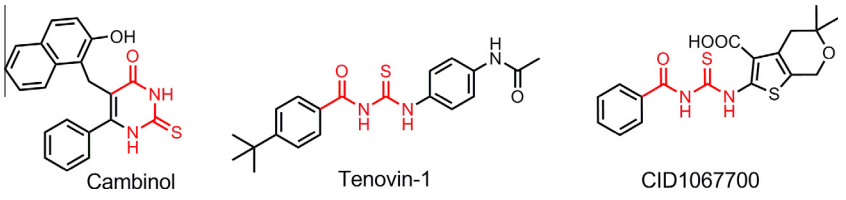
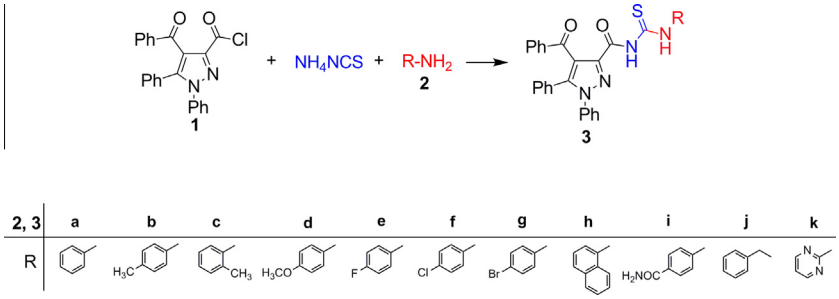
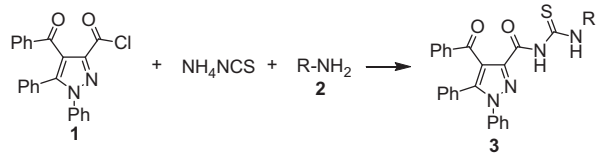


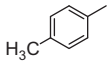
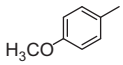
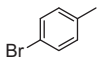
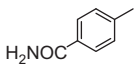
Figure 1. Structure of some bioactive acyl thiourea derivatives.



Scheme 1. The synthesis of pyrazolyl acyl thioureas.

Table 1
Some properties of synthesized compounds **3a–k**



Entry	R	Mp (°C)	Mol. Formula (Mol. wt.)	Yields		Micro analyses	
						Calcd (%)	Found (%)
3a		206	$C_{30}H_{22}N_4O_2S$ 502.59	79	C H N S	71.69 4.41 11.15 6.38	71.61 4.46 10.88 6.32
3b		216	$C_{31}H_{24}N_4O_2S$ 516.61	75	C H N S	72.07 4.68 10.85 6.21	71.82 4.82 10.55 6.23
3c		145	$C_{31}H_{24}N_4O_2S$ 516.61	72	C H N S	72.07 4.68 10.85 6.21	71.83 4.65 10.58 6.11
3d		199	$C_{31}H_{24}N_4O_3S$ 532.61	65	C H N S	69.91 4.54 10.52 6.02	69.70 4.41 10.26 5.94
3e		218	$C_{30}H_{21}FN_4O_2S$ 520.58	72	C H N S	69.22 4.07 10.76 6.16	69.13 4.22 10.48 6.16
3f		212	$C_{30}H_{21}ClN_4O_2S$ 537.03	69	C H N S	67.09 3.94 10.43 5.97	66.90 4.21 10.13 5.82
3g		204	$C_{30}H_{21}BrN_4O_2S$ 581.48	73	C H N S	61.97 3.64 9.64 5.51	62.24 3.92 9.54 5.28
3h		184	$C_{34}H_{24}N_4O_2S$ 552.65	68	C H N S	73.89 4.38 10.14 5.80	74.09 4.62 9.92 5.77
3i		245	$C_{31}H_{23}N_5O_3S$ 545.61	70	C H N S	68.24 4.25 12.84 5.88	68.02 4.06 12.69 5.51
3j		227	$C_{31}H_{24}N_4O_2S$ 516.61	71	C H N S	72.07 4.68 10.85 6.21	71.94 4.64 10.62 6.13
3k		219	$C_{28}H_{20}N_6O_2S$ 504.56	62	C H N S	66.65 4.00 16.66 6.36	66.37 3.78 16.32 6.33

activity at HepG2 cell lines. However, there is no significant impact of the halogenic side groups to the anticancer activity on the other cell lines used in this study.

Interestingly, addition of a single carbon to **3a** (which forms **3j**) increases anticancer activity significantly at DLD-1 cell lines (Fig. 3). **3a** (10^{-4} M), **3b** (10^{-4} M), **3d** (10^{-4} M), **3h**(10^{-4}, 10^{-5} M), and **3j** (10^{-4} M, 10^{-5} M) showed significant anticancer activity in Jurkat cell lines. The effect of different compounds to different types of cancer cell lines shows the importance of chemical structure on the cellular activity. It is likely that the pathway causing carcinoma at each cell line is different from each other; thus each cell line may have different targets for cancer prevention. Therefore, the compounds in this study exhibited a variety of impact on human colon, leukemia, and liver cell lines.

Surprisingly, **3a**, **3d**, and **3f** compounds at 48 h incubation displayed more cell viability compared to that of 24 h incubation at doses of 10^{-5} M concentration in Jurkat cell lines. This unexpected behavior may be explained as activation of transcription factor(s) of drug resistance mechanism at low doses of compounds as cited in the literature. Continuing proliferation of cells was also observed on cervical carcinoma cells as Jurkat cells.[40] In this study, it was shown that low doses of doxorubicin induce nuclear transcription factor-κB and this leads to drug resistance in SiHa cell lines (human cervical carcinoma cells). These results help drug design researchers to determine the effect of side groups at cellular level and provide a starting point to design effective anticancer compounds. Details of the unexpected behavior of these compounds may be highlighted by employing molecular methods.

Currently there are several drugs developed that target protein kinases for the treatment of different types of cancer. Small molecule kinase inhibitors are widely used in the pharmaceutical field such as, Axitinib®, Pazopanib®, Sunitinib® and Sorafenib®

Table 2
Anticancer activity of compounds as % cell viability after 24 and 48 h

Compounds	24 h			48 h		
	Jurkat cell line	DLD-1 cell line	HepG2 cell line	Jurkat cell line	DLD-1 cell line	HepG2 cell line
3a (10^{-4} M)	23.30 ± 2.31	71.35 ± 3.72	38.17 ± 1.47	6.00 ± 0.61	60.58 ± 3.46	21.84 ± 2.27
3a (10^{-5} M)	30.00 ± 1.19	75.54 ± 5.35	42.11 ± 3.61	67.60 ± 2.25	55.41 ± 3.78	23.73 ± 3.56
3b (10^{-4} M)	30.00 ± 3.25	57.14 ± 5.47	49.25 ± 5.88	12.46 ± 0.95	40.25 ± 3.91	25.91 ± 2.55
3b (10^{-5} M)	50.30 ± 6.33	65.14 ± 4.83	51.64 ± 4.65	14.20 ± 1.88	48.54 ± 4.44	27.71 ± 1.69
3c (10^{-4} M)	100.00 ± 5.87	55.28 ± 3.14	29.13 ± 1.22	76.59 ± 6.54	41.26 ± 4.21	20.81 ± 1.00
3c (10^{-5} M)	100.00 ± 3.77	63.24 ± 5.31	33.33 ± 2.13	51.18 ± 4.96	45.58 ± 3.87	22.82 ± 2.22
3d (10^{-4} M)	31.11 ± 3.1	71.25 ± 8.45	36.45 ± 4.13	8.98 ± 1.01	62.14 ± 6.54	22.64 ± 2.10
3d (10^{-5} M)	37.81 ± 3.67	80.45 ± 8.27	41.97 ± 5.33	87.50 ± 7.98	68.21 ± 7.64	27.87 ± 2.40
3e (10^{-4} M)	100.00 ± 6.4	62.45 ± 4.59	50.13 ± 5.13	67.60 ± 7.12	51.45 ± 4.1	23.56 ± 2.09
3e (10^{-5} M)	100.00 ± 6.1	75.14 ± 5.21	54.25 ± 4.31	47.80 ± 5.69	60.24 ± 6.25	30.06 ± 1.48
3f (10^{-4} M)	57.33 ± 5.66	60.45 ± 6.03	49.47 ± 3.12	24.20 ± 2.64	49.24 ± 5.17	24.78 ± 3.05
3f (10^{-5} M)	56.31 ± 5.41	73.29 ± 8.02	53.67 ± 5.21	90.00 ± 8.55	55.16 ± 4.44	24.94 ± 3.11
3g (10^{-4} M)	78.10 ± 7.77	65.25 ± 5.38	55.21 ± 5.16	40.10 ± 4.12	50.14 ± 5.55	27.19 ± 2.99
3g (10^{-5} M)	100.00 ± 2.10	78.12 ± 6.66	51.47 ± 4.19	87.50 ± 6.52	62.52 ± 6.07	27.79 ± 2.16
3h (10^{-4} M)	36.40 ± 4.25	74.25 ± 7.51	78.74 ± 5.11	8.24 ± 1.03	70.58 ± 4.15	49.85 ± 4.39
3h (10^{-5} M)	46.01 ± 5.01	82.15 ± 3.22	85.45 ± 2.18	14.53 ± 2.46	75.51 ± 4.37	51.24 ± 3.00
3i (10^{-4} M)	100.00 ± 3.56	100.00 ± 3.41	38.15 ± 2.18	39.24 ± 3.99	96.56 ± 8.51	22.94 ± 3.85
3i (10^{-5} M)	100.00 ± 2.11	100.00 ± 1.97	44.21 ± 4.17	66.35 ± 5.86	95.21 ± 7.41	27.26 ± 1.25
3j (10^{-4} M)	36.60 ± 4.25	64.15 ± 4.99	49.24 ± 4.51	17.87 ± 1.05	55.87 ± 3.14	39.77 ± 2.53
3j (10^{-5} M)	34.72 ± 2.50	75.15 ± 5.21	56.35 ± 6.54	23.13 ± 1.31	65.55 ± 6.58	40.63 ± 3.74
3k (10^{-4} M)	86.15 ± 4.44	80.44 ± 5.74	44.26 ± 3.51	23.42 ± 2.58	75.54 ± 5.41	28.80 ± 2.24
3k (10^{-5} M)	100.00 ± 1.32	88.54 ± 5.39	51.14 ± 4.87	27.98 ± 2.57	81.44 ± 5.56	31.24 ± 1.90

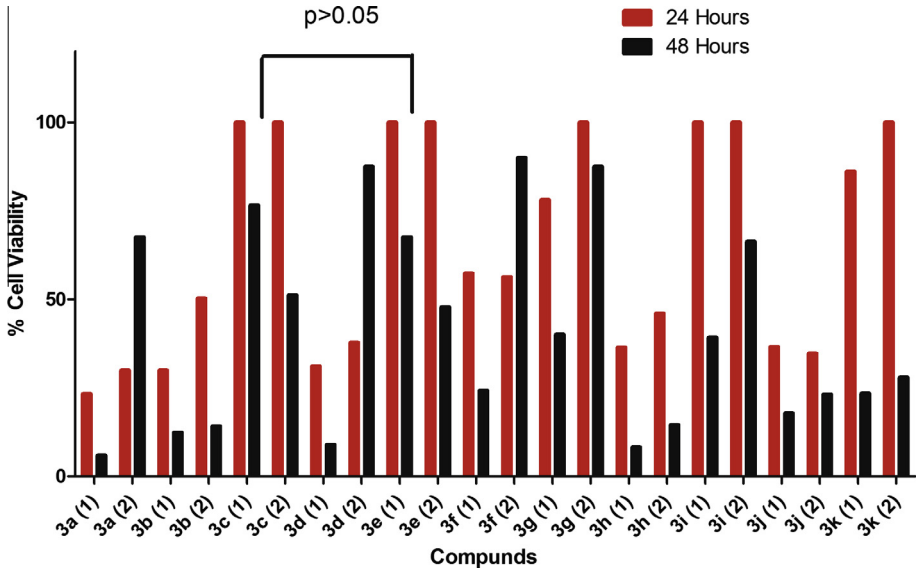


Figure 2. Antitumor activity of compounds for Jurkat cell line. (1) and (2) indicate 10^{-4} and 10^{-5} M respectively.

for renal cell carcinoma; Bosutinib® and Imatinib® for chronic myelogenous leukemia; Sunitinib®, Imatinib® for gastrointestinal stromal tumor; Lapatinib® for breast cancer, and Vemurafenib® for melanoma (www.fda.gov). In addition to these drugs, an excellent comprehensive review on small molecule kinase inhibitors explains several applications of these compounds.[41] Therefore, drug researchers have been focused on small molecule kinase inhibitors for affective cancer treatment.

Anti-tumoral activities of pyrazole rings and their derivatives have been known for many years. In pharmacy, cyclin-dependent kinases (CDKs) are considered a potential target for the development of anticancer drugs. CDKs are a family of protein kinases which play key role in regulating the cell cycle. Pyrazole derivatives generate anticancer action by the inhibition of the CDKs. CDK-mediated tumor cell proliferation may be inhibited in nanomolar concentrations of pyrazole based compounds. In the literature, 1-carboxamidopyrazole and aryl- and heteroaryl-substituted pyrazole derivatives show antiproliferative effects by inhibiting Aurora kinase activity and growth factor-beta type I receptor kinase respectively.[30,42] We can propose inhibition of CDKs as a model mechanism of our compounds for anticancer activities. Therefore, we determined anticancer characteristics of eleven small pyrazole derivative molecules in this study and we showed that these molecules have potential properties to be developed as kinase inhibitors.

3. Conclusion

We have synthesized a new series of acyl thiourea derivatives containing pyrazole ring were prepared in good yield via one pot reaction. The structures of these compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. Anticancer activities of synthesized compounds were evaluated on human leukemia, colon, and liver cancer cell lines. As a result of the cell culture studies, all of the compounds have shown anticancer activity for colon, leukemia

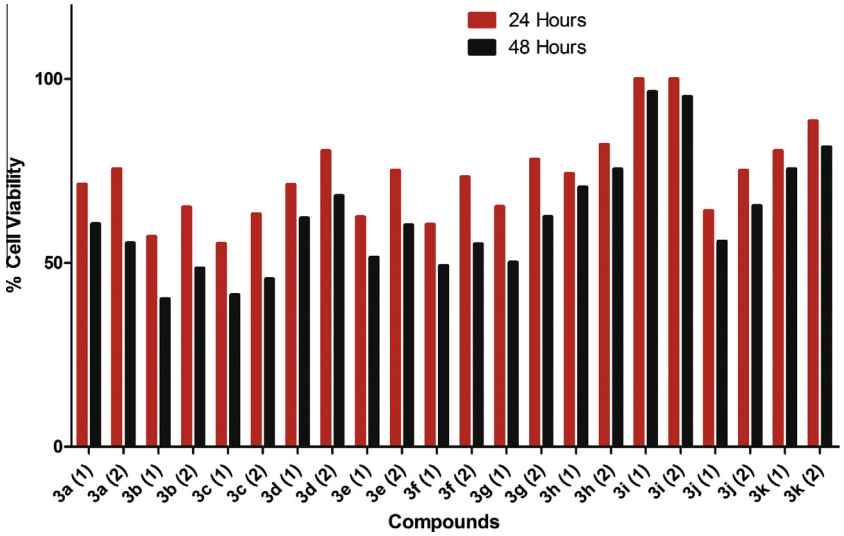


Figure 3. Antitumor activity of compounds for DLD-1 cell line. (1) and (2) indicate 10^{-4} and 10^{-5} M respectively.

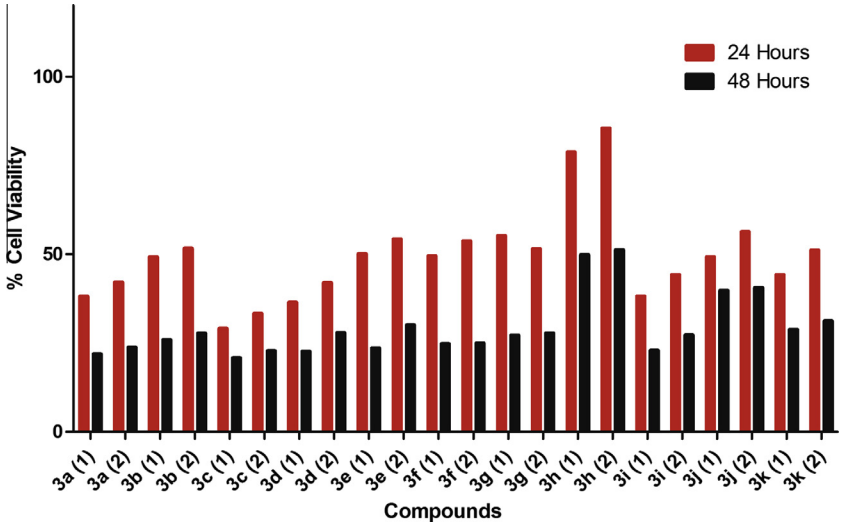


Figure 4. Antitumor activity of compounds for HepG2 cell line. (1) and (2) indicate 10^{-4} and 10^{-5} M respectively.

and liver cancer cells. In conclusion, novel pyrazolyl acyl thioureas compounds might be potentially useful in the field of cancer treatment. Finally, our new synthesized compounds can be suggested as potent candidates for leukemia, liver and colon cancer drugs.

4. Experimental section

4.1. Chemistry

Melting points are uncorrected and recorded on Electrothermal 9200 digital melting point apparatus. Microanalyses were performed on a Leco-932 CHNS-O Elemental Analyser. A Perkin Elmer Spectrum Two Model FT-IR Spectrophotometer was used for IR spectra using ATR method. The ^{1}H and ^{13}C NMR spectra were measured with Bruker Avance III 400 MHz spectrometer using $CDCl_3$ solvent. The reactions were followed by TLC (Silica gel, aluminium sheets 60 F_{254}, Merck). Solvents and all other chemical reagents were purchased from Merck, Sigma, Aldrich and Fluka. Solvents were dried by refluxing with the appropriate drying agents and distilled before use. DLD-1, HepG2 and Jurkat cell lines were supplied from ATCC (American Type Culture Collection, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and sterile phosphate buffer saline (PBS) were obtained from PAA Ltd. (France). Trypsin–EDTA was from Biological Industries Ltd. (Haemek, Israel). L-glutamine–penicillin–streptomycin solution was from Sigma–Aldrich (Steinheim am Albuch, Germany). XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) was obtained from Roche Diagnostic.

4.2. General procedure for the synthesis of compound 3

0.387 g (1 mmol) 4-benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carbonyl chloride (**1**) and 0.084 g (1.1 mmol) ammonium thiocyanate in 15 mL acetone were refluxed for 30 min. The resulting solid (NH_4Cl) was removed by filtration. Then to this solution corresponding amines (1 mmol) in 10 mL acetone was added drop wise, the mixture was stirred under reflux for 3 h. The solvent was removed under reduced pressure, and the residue was treated with the 2-propanol to give crude product as a white solid. The resulting precipitates were purified by column chromatography on silica gel using chloroform/hexane (1:1) as eluent.

4.2.1. 4-Benzoyl-1,5-diphenyl-*N*-(phenylcarbamothioyl)-1*H*-pyrazole-3-carboxamide (3a)

Yield 0.397 g, 79%, mp 206 °C. IR (ATR, cm^{-1}): 3191 (b, NH), 1682, 1665 (C=O), 1176 (C=S). ^1H NMR ($CDCl_3$, ppm): 12.06 (s, 1H, NH), 10.02 (s, 1H, NH), 7.88–7.16 (m, 20H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.99 (C=O), 178.07 (C=S), 159.97 (C=O, amide), 144.52, 142.12, 138.39, 137.59, 137.30, 133.74, 129.64, 129.53, 129.25, 129.05, 128.77, 128.73, 128.61, 127.33, 126.81, 125.30, 124.30, 123.10 (C=C and C=N). Calcd for $C_{30}H_{22}N_4O_2S$ (502.59): C, 71.69; H, 4.41; N, 11.15; S, 6.38. Found: C, 71.61; H, 4.46; N, 10.88; S, 6.32.

4.2.2. 4-Benzoyl-1,5-diphenyl-*N*-(*p*-tolylcarbamothioyl)-1*H*-pyrazole-3-carboxamide (3b)

Yield 0.386 g, 75%, mp 216 °C. IR (ATR, cm^{-1}): 3191 (b, NH), 1682, 1665 (C=O), 1176 (C=S). ^1H NMR ($CDCl_3$, ppm): 11.95 (s, 1H, NH), 9.99 (s, 1H, NH), 7.87–7.16 (m, 19H, Ar-H), 2.34 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, ppm): 190.98 (C=O), 178.17 (C=S), 159.92 (C=O, amide), 144.47, 142.16, 138.40, 137.31, 136.75, 135.03, 133.71, 129.64, 129.52, 129.23, 129.02, 128.72, 128.59, 127.36, 125.29, 124.36, 123.08 (C=C and C=N), 21.08(CH_3). Calcd for $C_{31}H_{24}N_4O_2S$ (516.61): C, 72.07; H, 4.68; N, 10.85; S, 6.21. Found: C, 71.82; H, 4.82; N, 10.55; S, 6.23.

4.2.3. 4-Benzoyl-1,5-diphenyl-*N*-(*o*-tolylcarbamothioyl)-1*H*-pyrazole-3-carboxamide (3c)

Yield 0.372 g, 72%, mp 145 °C. IR (ATR, cm^{-1}): 3134 (b, NH), 1674, 1662 (C=O), 1173 (C=S). ^1H NMR ($CDCl_3$, ppm): 11.70 (s, 1H, NH), 10.11 (s, 1H, NH), 7.87–7.16 (m, 19H, Ar-H), 2.24 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, ppm): 190.97 (C=O), 179.25 (C=S), 160.07 (C=O, amide), 144.64, 142.26, 138.39, 137.40, 136.32, 133.82, 133.66, 130.72, 129.68, 129.65, 129.50, 129.24, 129.04, 128.72, 128.54, 127.75, 127.35, 126.58, 126.41, 125.29, 123.03 (C=C and C=N), 17.91 (CH_3). Calcd for $C_{31}H_{24}N_4O_2S$ (516.61): C, 72.07; H, 4.68; N, 10.85; S, 6.21. Found: C, 71.83; H, 4.65; N, 10.58; S, 6.11.

4.2.4. 4-Benzoyl-*N*-(*4*-methoxyphenylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3d)

Yield 0.346 g, 65%, mp 199 °C. IR (ATR, cm^{-1}): 3177 (b, NH), 1678, 1669 (C=O), 1186 (C=S). ^1H NMR ($CDCl_3$, ppm): 11.89 (s, 1H, NH), 10.00 (s, 1H, NH), 7.87–6.88 (m, 19H, Ar-H), 3.81 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$, ppm): 191.00 (C=O), 178.39 (C=S), 159.95 (C=O, amide), 158.18, 144.46, 142.15, 138.39, 137.30, 133.73, 130.54, 129.63, 129.52, 129.23, 129.03, 128.73, 128.61, 127.35, 126.03, 125.29, 123.08, 113.98 (C=C and C=N), 55.43 (OCH_3). Calcd for $C_{31}H_{24}N_4O_3S$ (532.61): C, 69.91; H, 4.54; N, 10.52; S, 6.02. Found: C, 69.70; H, 4.41; N, 10.26; S, 5.94.

4.2.5. 4-Benzoyl-*N*-(*4*-fluorophenylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3e)

Yield 0.375 g, 72%, mp 218 °C. IR (ATR, cm^{-1}): 3146 (b, NH), 1676, 1622 (C=O), 1181 (C=S). ^1H NMR ($CDCl_3$, ppm): 11.98 (s, 1H, NH), 10.03 (s, 1H, NH), 7.87–7.04 (m, 19H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.96 (C=O), 178.60 (C=S), 160.93 (d, ^1J = 248.0 Hz, C–F), 160.06 (C=O, amide), 144.54, 142.01, 138.37, 137.26, 133.76, 133.58, 133.55, 129.67, 129.62, 129.53, 129.26, 129.08, 128.74, 128.62, 127.29, 126.46, 126.37, 125.29, 123.12, 115.65 (d, ^2J = 23.0 Hz), (C=C and C=N). Calcd for $C_{30}H_{21}FN_4O_2S$ (520.58): C, 69.22; H, 4.07; N, 10.76; S, 6.16. Found: C, 69.13; H, 4.22; N, 10.48; S, 6.16.

4.2.6. 4-Benzoyl-*N*-(*4*-chlorophenylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3f)

Yield 0.372 g, 69%, mp 212 °C. IR (ATR, cm^{-1}): 3161 (b, NH), 1679, 1622 (C=O), 1188 (C=S). ^1H NMR ($CDCl_3$, ppm): 12.09 (s, 1H, NH), 10.04 (s, 1H, NH), 7.87–7.15 (m, 19H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.92 (C=O), 178.14 (C=S), 160.05 (C=O, amide), 144.57, 141.98, 138.37, 137.26, 136.14, 133.75, 132.05, 129.68, 129.63, 129.53, 129.25, 129.09, 128.88, 128.74, 128.61, 127.29, 125.50, 125.30, 123.14 (C=C and C=N). Calcd for $C_{30}H_{21}ClN_4O_2S$ (537.03): C, 67.09; H, 3.94; N, 10.43; S, 5.97. Found: C, 66.90; H, 4.21; N, 10.13; S, 5.82.

4.2.7. 4-Benzoyl-*N*-(*4*-bromophenylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3g)

Yield 0.342 g, 73%, mp 204 °C. IR (ATR, cm cm^{-1}): 3191 (b, NH), 1682, 1665 (C=O), 1176 (C=S). ^1H NMR ($CDCl_3$, ppm): 12.08 (s, 1H, NH), 10.03 (s, 1H, NH), 7.96–7.16 (m, 19H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.91 (C=O), 178.04 (C=S), 160.05 (C=O, amide), 144.58, 141.98, 138.38, 137.27, 136.67, 133.75, 131.85, 129.68, 129.63, 129.53, 129.25, 129.09, 128.74, 128.60, 128.33, 127.30, 125.74, 125.31, 123.16, 119.89 (C=C and C=N). Calcd for $C_{30}H_{21}BrN_4O_2S$ (581.48): C, 61.97; H, 3.64; N, 9.64; S, 5.51. Found: C, 62.24; H, 3.92; N, 9.54; S, 5.28.

4.2.8. 4-Benzoyl-*N*-(naphthalen-1-ylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3h)

Yield 0.376 g, 68%, mp 184 °C. IR (ATR, cm^{-1}): 3130 (b, NH), 1677, 1669 (C=O), 1185 (C=S). ^1H NMR ($CDCl_3$, ppm): 11.95 (s, 1H, NH), 9.99 (s, 1H, NH), 7.87–7.16 (m, 22H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.99 (C=O), 179.95 (C=S), 160.25 (C=O, amide), 144.73, 142.29, 138.41, 137.45, 134.13, 133.67, 133.58, 129.71, 129.68, 129.53, 129.26, 129.07, 128.74, 128.66, 128.57, 128.48, 128.02, 127.35, 126.82, 126.34, 125.32, 125.18, 124.27, 123.11, 121.98 (C=C and C=N). Calcd for $C_{34}H_{24}N_4O_2S$ (552.65): C, 73.89; H, 4.38; N, 10.14; S, 5.80. Found: C, 74.09; H, 4.62; N, 9.92; S, 5.77.

4.2.9. 4-Benzoyl-*N*-(*4*-carbamoylphenylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3i)

Yield 0.380 g, 70%, mp 245 °C. IR (ATR, cm^{-1}): 3422, 3377 (NH_2), 3191(b, NH), 1682, 1665 (C=O), 1176 (C=S). ^1H NMR ($CDCl_3$, ppm): 11.95 (s, 1H, NH), 9.99 (s, 1H, NH), 7.87–7.16 (m, 19H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.95 (C=O), 177.80 (C=S), 168.29 (C=O, amide), 160.07 (C=O, amide), 144.60, 141.91, 140.87, 138.35, 137.21, 133.80, 131.02, 129.69, 129.61, 129.54, 129.27, 129.11, 128.75, 128.62, 128.07, 127.25, 125.31, 123.55, 123.15 (C=C and C=N). Calcd for $C_{31}H_{23}N_5O_3S$ (545.61): C, 68.24; H, 4.25; N, 12.84; S, 5.88. Found: C, 68.02; H, 4.06; N, 12.69; S, 5.51.

4.2.10. 4-Benzoyl-*N*-(benzylcarbamothioyl)-1,5-diphenyl-1*H*-pyrazole-3-carboxamide (3j)

Yield 0.367 g, 71%, mp 227 °C. IR (ATR, cm^{-1}): 3246 (b, NH), 1679, 1660 (C=O), 1190 (C=S). ^1H NMR ($CDCl_3$, ppm): 10.56 (s, 1H, NH), 9.95 (s, 1H, NH), 7.82–7.13 (m, 20H, Ar-H), 4.86 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$, ppm): 190.97 (C=O), 179.76 (C=S), 159.81 (C=O, amide), 144.39, 142.24, 138.39, 137.22, 136.16, 133.66, 129.61, 129.51, 129.21, 128.99, 128.73, 128.69, 128.54, 127.93, 127.80, 127.36, 125.99, 122.93 (C=C and C=N), 49.60 (CH_2–N). Calcd for $C_{31}H_{24}N_4O_2S$ (516.61): C, 72.07; H, 4.68; N, 10.85; S, 6.21. Found: C, 71.94; H, 4.64; N, 10.62; S, 6.13.

4.2.11. 4-Benzoyl-1,5-diphenyl-*N*-(pyrimidin-2-ylcarbamothioyl)-1*H*-pyrazole-3-carboxamide (3k)

Yield 0.313 g, 62%, mp 219 °C. IR (ATR, cm^{-1}): 3196 (b, NH), 1720, 1667 (C=O), 1193 (C=S). ^1H NMR ($CDCl_3$, ppm): 8.68 (s, 2H, 2 × NH), 7.87–7.07 (m, 18H, Ar-H). ^{13}C NMR ($CDCl_3$, ppm): 190.81 (C=O), 176.80 (C=S), 159.02 (C=O, amide), 158.17, 157.26, 144.26, 138.71, 137.49, 133.54, 129.64, 129.57, 129.19, 128.90, 128.70, 128.51, 127.41, 125.21, 123.45, 117.18 (C=C and C=N). Calcd for $C_{28}H_{20}N_6O_2S$ (504.56): C, 66.65; H, 4.00; N, 16.66; S, 6.36. Found: C, 66.37; H, 3.78; N, 16.32; S, 6.33.

4.3. In vitro studies

4.3.1. Cell lines and culture

For in vitro experiments, DLD-1 (human colorectal adenocarcinoma), HepG2 (human hepatocellular carcinoma) and Jurkat (human acute T-cell leukemia) cell lines were cultured in DMEM (low glucose) medium with 10% fetal bovine serum, 1% l-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin. Cells were cultivated in a humidified incubator at 37 °C within an atmosphere containing 5% CO_2.

4.3.2. Cytotoxic assay of compounds

XTT test was used to monitor pyrazolyl acyl thioureas derivatives toxicity on cancer cells. Initially, the cancer cells were placed in sterile 96-well culture plate (10×10^4 cells in each well), and our compounds were tested at individual experiments by 10-fold diluting the starting mixture at five different concentration ranging from 10^{-4} to 10^{-8} M at 24 and 48 h. At the end of these periods, 50 μl XTT reagents were added to each well for determination of living cells. The mitochondrial dehydrogenase enzymes reduce yellow colored tetrazolium salt (XTT) into water-soluble orange colored formazan salt. After 4 h, the absorbance was measured using micro plate reader (Thermo) at 450 nm, and then the percentage of cell viability was calculated.[38,39]

4.3.3. Statistical analysis

Differences in the mean values of measured activities were evaluated statistically using the SPSS 17.0 program (Univariate Variance Analyses and Pearson Correlation). Probability values of $p < 0.05$ were considered to be significant.

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