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Article

Anticancer Activity Evaluation of New Thieno[2,3-*d*]pyrimidin-4(3*H*)-ones and Thieno[3,2-*d*]pyrimidin-4(3*H*)-one Derivatives

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Abstract: Anticancer screening of several novel thienopyrimidines has been performed. The thienopyrimidine derivatives were synthesized from available starting materials according to the convenient synthetic procedures using a one-pot solvent-free reaction which gave a wide access to thienopyrimidine-derivative production. The synthesized compounds were preselected via molecular docking to be tested for their anticancer activity in NCI 60 cell lines. It was observed that some compounds showed remarkable anticancer activity. It was found that the most active compound among thieno[2,3-*d*]pyrimidine-4(3*H*)- ones is 2-(benzylamino)-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one, which possesses cytotoxic activity on almost all cancer cell lines with mean growth 51.01%, where the most sensitive was the melanoma cell line MDA-MB-435 with GP (Growth Percent) = −31.02%. The patterns of structure–activity that are important for further optimization of the structure and the creation of more selective and active anticancer agents were proposed.

Keywords: anticancer activity; 2-R^3,R^4-amino-5-R^1-6-R^2-thieno[2,3-*d*]pyrimidin-4(3*H*)-ones; 2-R^3, R^4-amino-5-R^1-6-R^2-thieno[3,2-*d*]pyrimidin-4(3*H*)-ones; molecular docking

1. Introduction

From the standpoint of pharmacological activity, thienopyrimidine derivatives continue to attract great interest due to the wide variety of interesting biological activities ([1,2] and articles cited therein). Thienopyrimidines are structural analogues of biogenic purines and can be considered as potential nucleic acid antimetabolites. Therefore, they occupy a special position among fused pyrimidines, along with some other pyrimidines containing an annulated five-membered heteroaromatic ring.

The epidermal growth factor receptor tyrosine kinase (EGFR) plays an important role in carcinogenesis and is therefore an intriguing target for cancer therapy [3]. EGFR is an attractive target in EGFR-driven diseases such as non-small cell lung cancer [4,5] EGFR-positive breast cancer [6] and in pancreatic cancer [7]. In reality, the larger part of human epithelial tumors, including lung cancer, ovarian tumor and breast cancer, are associated with functional activation of the EGFR family receptors and growth factors. Thus, selective blockade of EGFR has been shown to be an effective therapeutic approach against multiple epithelial cancers [4]. Scaffolds employed include mainly quinazolines, pyrrolopyrimidines and furopyrimidines [3]. Additionally, scientists have also identified potent thienopyrimidine-based EGFR inhibitors [3].

Moreover, at least three compounds with the thienopyrimidine scaffold (Figure 1) have been developed and investigated for anticancer activity by such pharmaceutical companies of global renown as Genentech, Pyramid and Sunesis Pharmaceuticals [8–10]. They are already being tested in the clinical trial (Phase 1, Phase 1 and Phase 2, respectively).

The present work is devoted to the synthesis and evaluation of anticancer activity of new substituted thienopyrimidines. The point of the research was to perform in-vitro anticancer activity assays of the most active thienopyrimidines based on the results obtained via computer simulation—molecular docking. The last one was used for the purposeful searching of EGFR tyrosine kinase inhibitors as potential anticancer agents. The structures shown in the article were preselected from a number of substructure molecules.

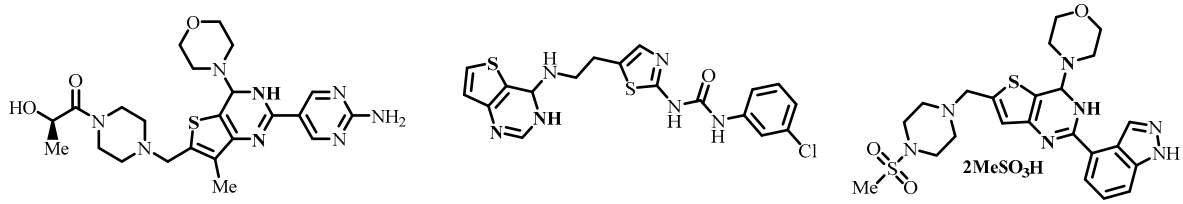


Figure 1. Structure of potent thienopyrimidine-based lead compounds developed by pharmaceutical companies worldwide.

2. Materials and Methods

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury 400 (400 MHz for ^{1}H), man. Agilent Technologies Inc. and on a Bruker 500 (126 MHz for ^{13}C), man. Bruker Inc. instrument with TMS (Tetramethylsilane) or deuterated solvent as an internal reference. Mass spectra were run using Agilent 1100 series LC/MSD, man. Agilent Technologies Inc. with an API–ES/APCI ionization mode. Satisfactory elemental analyses were obtained for new compounds (C $\pm$ 0.17, H $\pm$ 0.21, N $\pm$ 0.19).

Materials for MTT assay:

A 10 mM stock solution of thienopyrimidine derivatives was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), and additionally dissolved in culture medium prior to addition to the cell culture. In-vitro screening of the anticancer action of the synthesized compounds and doxorubicin towards tumor cell was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test (99.5% pure, Sigma-Aldrich, St. Louis, MO, USA). Cell culture medium DMEM was obtained from Sigma-Aldrich, St. Louis, MO, USA; RPMI-1640—from PPA, Vienna, Austria. Fetal bovine serum (FBS) was obtained from Biowest, Nuaille, France. Doxorubicin was obtained from Pharmachemie B.V., Haarlem, The Netherlands.

Cell cultures for MTT assay:

Human breast adenocarcinoma cells of MCF-7 line, human lung adenocarcinoma cells of A549 line, human glioblastoma cells of U251 line, human melanoma cells of WM793 line, human myeloid leukemia cells of K562 line, human acute T-cell leukemia cells of Jurkat and human embryonic kidney cells of HEK293 line were obtained from collection at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiology (Kyiv, Ukraine). Cells were grown in the RPMI-1640 or DMEM culture medium supplemented with 10% of fetal bovine serum. Cells were cultivated in the CO_2-thermostate at 37 °C in atmosphere of 95% air and 5% CO_2.

2.1. Chemistry

Compounds **1a**–**f** were prepared according to the methods of Gewald et al. [11,12].

General procedure for the synthesis of 1*H*-tetrazol-1-yl thiophene-carboxylates **2**.

A suspension of 50 mmol of the required aminothiophene, triethyl orthoformate (37.9 mL, 0.23 mol), and sodium azide (3.9 g, 0.06 mol) in glacial acetic acid (40 mL) was stirred and heated at reflux for 2 h. The reaction mixture was cooled to room temperature and 7 mL of conc. HCl was added. The solid was filtered off and the filtrate evaporated and the residue was recrystallized from ethanol.

General procedure for the synthesis of thieno[2,3-*d*]pyrimidine-4(3*H*)-one and thieno[3,2-*d*] pyrimidine-4(3*H*)-one derivatives **4**–**6**.

A suspension of an appropriate tetrazole **2** (1 mmol) in 0.8 mL of the corresponding amine **3** was heated at 80–90 °C for 0.5–1 h, then cooled and diluted with water (1 mL). The solid was filtered and recrystallized from ethanol.

2.2. Pharmacology

2.2.1. Anticancer Assay via NCI Protocol

Accordingly, to the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda, Maryland, a primary anticancer assay was performed within nine cancer types on a panel of approximately 60 human tumor cell lines. The tested compounds were added to the culture at a single concentration (10^{-5} M) and left for 48 h incubation. Sulforhodamine B (SRB) was used as protein binding dye for the end-point determinations. The percent of growth of the treated cells when compared to the untreated control cells was taken as a result for each tested compound. The percentage growth was evaluated spectrophotometrically versus controls not treated with test agents.

2.2.2. Cell Proliferation (MTT) Assay

In-vitro evaluation of anticancer activity of the synthesized compounds and doxorubicin, used as a reference drug control, towards cancer cell lines was measured by the MTT test [13]. Tumor cells were seeded for 24 h in 96-well microtiter plates at a concentration of 5000 substrate-dependent cells/well or 10,000 suspension cells/well (100 µL/well); after that, cells were incubated for 72 h with various additions of the synthesized compounds (0–50 µM). MTT, which is converted to dark blue, water-insoluble MTT formazan by the mitochondrial dehydrogenases, was used to determine viable cells according to the Sigma-Aldrich protocol. The IC_{50} of the tested compounds was calculated as a concentration of drug killing 50% of cells in comparison with an untreated culture.

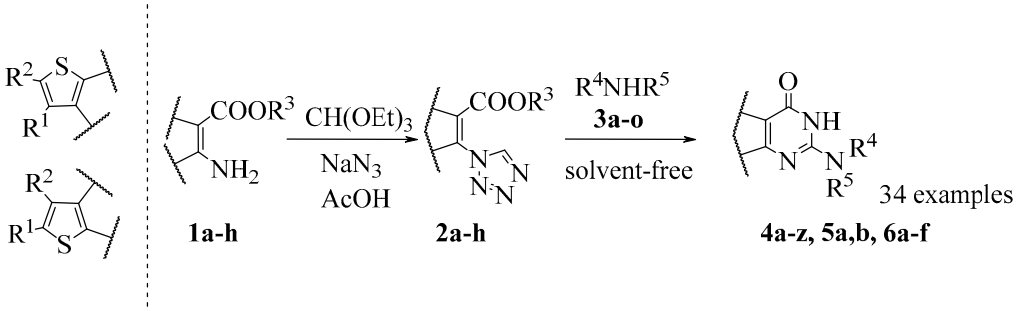
2.2.3. Statistical Analysis

All data are presented as the mean (M) ± standard deviation (SD). Results were analyzed and illustrated with GraphPad Prism (version 6; GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using two-way ANOVA with Bonferroni post-tests (tumor growth). A *p*-value of <0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Chemistry

The compounds presented in the article were obtained in a simple and convenient synthetic path (Scheme 1). The Gewald thiophenes and isomeric 3-aminothiophenes **1a**–**h** were used as starting materials in the synthesis of thienopyrimidine derivatives. Alkyl amino-thiophene-carboxylates were converted into corresponding alkyl 3-(1*H*-tetrazol-1-yl)-4-R^1-5-R^2-thiophene-2-carboxylates **2a**–**c** and alkyl 2-(1*H*-tetrazol-1-yl)-4-R^1-5-R^2-thiophene-3-carboxylates **2d**–**h** by the reaction with triethyl orthoformate and sodium azide with good yields according to previously reported synthetic protocol [14–16]. It was found that obtained thienyl-tetrazoles **2a**–**h** after their treatment with aliphatic amines **3** underwent recyclization including cleavage of the tetrazole ring, elimination of the nitrogen molecule and annulation of the pyrimidinone core under one-pot solvent-free conditions (Scheme 1).



Scheme 1. Synthesis of 2-R^3,R^4-amino-5-R^1-6-R^2-thieno[3,2-*d*]pyrimidin-4(3*H*)-ones **6a**–**f** and 2-R^3,R^4-amino-5-R^1-6-R^2-thieno[2,3-*d*]pyrimidin-4(3*H*)-ones **4a**–**z**, **5a**,**b** derivatives.

This versatile, convenient, efficient and high-yield synthetic method allowed us to prepare 28 examples of 2-R^3,R^4-amino-5-R^1-6-R^2-thieno[2,3-*d*]pyrimidin-4(3*H*)-ones and 2-R^3,R^4-amino-5-R^1-6-R^2-thieno[3,2-*d*]pyrimidin-4(3*H*)-ones **4a**–**z**, **5a**,**b**, **6a**–**f** as described in the literature [15,16].

To perform anticancer activity evaluation and in order to broaden a diversity of thieno[2,3-*d*]pyrimidine and thieno[3,2-*d*]pyrimidine derivatives with substituents of different nature, some new examples (**4e**,**g**,**j**,**w**,**z** and **6a**,**c**,**f**) of such compounds are reported herein for the first time. It should be noted that overall yields for the desired thienopyrimidines were 72–88%, which allowed us quickly and without chromatographic purification to create a library of such compounds. A variety of amines **3** successfully used in such a protocol are shown in Figure 2.

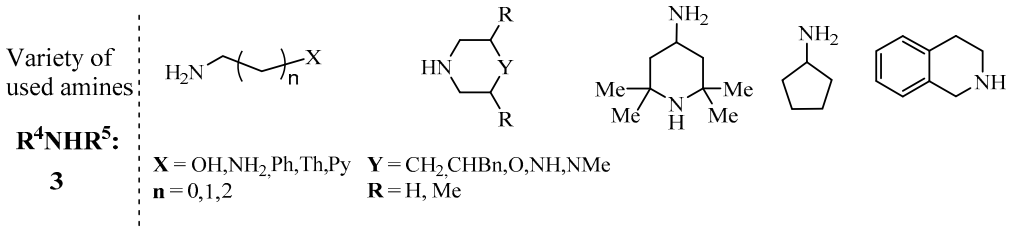


Figure 2. A variety of successfully used amines in the synthesis of thieno[3,2-*d*]pyrimidin-4(3*H*)-one thieno[2,3-*d*]pyrimidin-4(3*H*)-one **5a**–**z** derivatives.

3.2. Molecular Docking

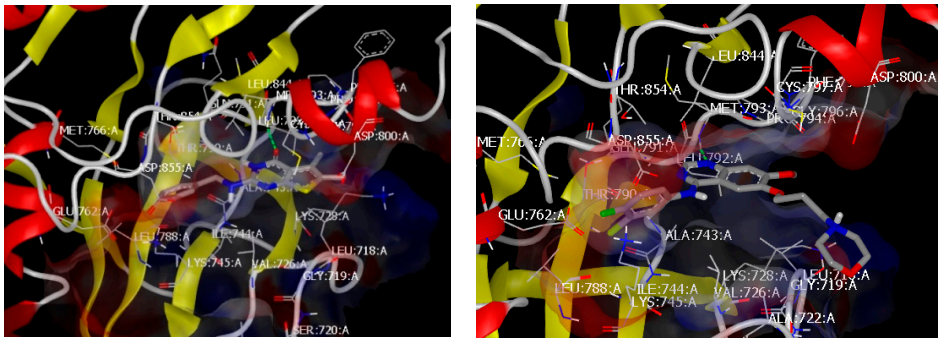
Molecular docking was conducted with OpenEye Scientific Software program package as a computer method approach to the search of molecules with affinity to certain biotargets. Used software includes Fred Receptor 2.2.5, Vida 4.1.0, Flipper, Babel3, Omega2 and Fred2 programs. Chrystallographic model of EGFR tyrosine kinase (2ITY) was obtained from Protein Data Bank (www.rcsb.org). As research objects, obtained thienopyrimidine derivatives and well-known selective EGFR inhibitors, such as erlotinib, lapatinib, gefitinib and others, were chosen. To estimate *in silico* EGFR tyrosine kinase–compound binding, seven scoring function values (chemgauss2, chemscore, PLP, screenscore, shapegauss, zapbind and consensus) were calculated. Cumulative (consensus) scoring function ranking allowed us to select compounds that could prospectively be selective EGFR tyrosine kinase inhibitors at the level of erlotinib for future (in-depth) pharmacological studies, as well as could be used as templates for the synthesis of various related analogues. The Fred receptor program allows us to extract the active site (biotarget) of EGFR tyrosine kinase from a crystallographic model for molecular docking.

Molecular docking studies included a generation of R-, S- and cis–trans isomers of ligands using the program Flipper with further 3D optimization of isomers using the program Hyper Chem 7.5 (www.hyper.com) (molecular mechanics method MM+ and semi-empirical quantum-mechanical

method PM3). Conformers were generated via Omega2. A further program, Fred2, chose minimum energy conformations for each molecule, and 3D molecular docking was performed.

Values of the seven scoring functions (chemgauss2, chemscore, PLP, screenscore, shapegauss, zapbind and consensus) were obtained as a result. Ranking property (compound ranking) of the consensus scoring function, which includes values of all scoring functions, allowed us to analyze the results easily.

Ranking and analysis of the molecular docking results were obtained using the selected compounds and crystallographic model of EGFR tyrosine kinase with cumulative scoring function (consensus) that allowed us to select 34 compounds for further evaluation of in-vitro anticancer activity. The interactions between EGFR tyrosine kinase active site and the most active compound **5a** in comparison with selective inhibitor gefitinib is shown in the Figure 3. Moreover, it should be noted that results predicted via docking correlate quite well with those obtained in the in-vitro assay, especially while talking about activity against non-small cell lung cancer cell lines (Table 1 and Table S1). The selected "lead" compound **5a** based on the in-vitro screening results was also predicted to be the most active in the docking studies.



(**a**) (**b**)

Figure 3. Compound **5a** docked in the active site of EGFR tyrosine kinase (**a**), in comparison with selective inhibitor gefitinib docked in the active site of EGFR tyrosine kinase (**b**).

Table 1. Anticancer screening data in concentration 10^{-5} M for the most active thienopyrimidines.

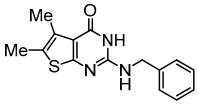
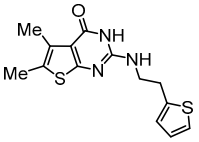
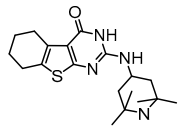
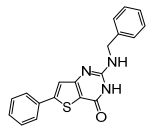
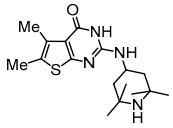
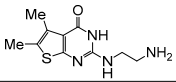
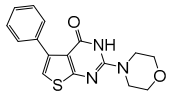
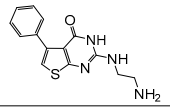
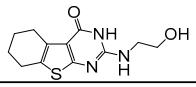
No.	Compound	Mean Growth %	Range of Growth %	The Most Sensitive Cell Lines	Growth of the Most Sensitive Cell Lines
5a		51.01	−31.02 to 91.62	MDA-MB-435 (Melanoma) K-562 (Leukemia) HT 29 (Colon Cancer) SR (Leukemia) HL-60 (TB) (Leukemia) MDA-MB-468 (Breast Cancer) HCT 116 (Colon Cancer) HT SW-620 (Colon Cancer) NCI-H460 (Non-Small Cell Lung Cancer)	−31.02 12.90 13.28 14.44 22.45 23.78 24.50 25.96 26.58
4x		85.98	58.71 to 105.80	A549/ATCC (Non-Small Cell Lung Cancer) HCT-15 (Colon Cancer) UACC-62 (Melanoma) HOP-92 (Non-Small Cell Lung Cancer) A498 (Renal Cancer) PC-3 (Prostate Cancer)	58.71 59.57 63.06 68.44 70.69 71.86
4s		88.10	51.19 to 105.87	HOP-92 (Non-Small Cell Lung Cancer) SNB-75 (CNS Cancer) U251 (CNS Cancer) MOLT-4 (Leukemia) PC-3 (Prostate Cancer) MDA-MB-468 (Breast Cancer)	51.19 70.21 71.86 72.91 73.10 74.47

Table 1. *Cont.*

No.	Compound	Mean Growth %	Range of Growth %	The Most Sensitive Cell Lines	Growth of the Most Sensitive Cell Lines
6a		91.38	58.12 to 110.00	HOP-92 (Non-Small Cell Lung Cancer) MALME-3M (Melanoma) NCI/ADR-RES (Ovarian Cancer) NCI-H322M (Non-Small Cell Lung Cancer) UO-31 (Renal Cancer)	58.12 74.98 78.18 78.19 79.54
4r		92.97	66.09 to 113.41	SNB-75 (CNS Cancer) HOP-92 (Non-Small Cell Lung Cancer) MALME-3M (Melanoma) BT-549 (Breast Cancer) UO-31 (Renal Cancer)	66.09 67.24 73.78 76.08 77.53
4c		93.98	67.36 to 117.18	SNB-75 (CNS Cancer) COLO 205 (Colon Cancer) MDA-MB-468 (Breast Cancer)	67.36 76.97 80.04
4m		95.06	53.30 to 110.21	EKVX (Non-Small Cell Lung Cancer) U251 (CNS Cancer) NCI-H522 (Non-Small Cell Lung Cancer) HOP-92 (Non-Small Cell Lung Cancer)	53.30 69.49 76.95 79.72
4d		97.43	59.72 to 129.11	EKVX (Non-Small Cell Lung Cancer) SNB-75 (CNS Cancer) HOP-92 (Non-Small Cell Lung Cancer) MDA-MB-468 (Breast Cancer)	59.72 63.87 74.21 78.04
4b		99.06	43.50 to 126.21	EKVX (Non-Small Cell Lung Cancer) MDA-MB-468 (Breast Cancer) SNB-75 (CNS Cancer)	43.50 84.23 85.73

3.3. Evaluation of Anticancer Activity In Vitro

The synthesized thienopyrimidines (**4a**–**z**, **5a**,**b**, **6a**–**f**) were submitted and evaluated at the single concentration of 10^{-5} M towards a panel of the approximately sixty cancer cell lines. The human tumor cell lines were derived from the nine different cancer types: leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancers. Primary anticancer assays were performed according to the US NCI protocol, which was described elsewhere [17–20]. The results for each compound are reported as the percent growth (GP) (Table S1). Range of growth (%) shows the lowest and the highest growth that was found among different cancer cell lines.

The synthesized thienopyrimidines displayed moderate or low activity in the in-vitro screen on tested cell lines. The most active compounds are presented in Table 1. However, a selective influence of some compounds on several cancer cell lines was observed. The compounds **4s** and **6c** were quite active on non-small cell lung cancer HOP-92 cell line (GP = 51.19% and GP = 58.12%, respectively), and the compounds **4b**,**m**,**d** were quite active on the non-small cell lung cancer EKVX cell line (GP = 43.50%, GP = 53.30% and GP = 59.72%, respectively). The compound **4x** was quite active on non-small cell lung cancer A549/ATCC cell line (GP = 58.71%) and active on colon cancer HCT-15 cell line (GP = 59.57%) The majority of the tested compounds displayed growth inhibition on prostate cancer cell line PC-3 (**4e**,**f**,**h**,**k**,**l**,**n**,**s**,**t**,**x**,**z**, **6f**), CNS cancer cell line SNB-75 (**6b**,**d**,**e**, **4b**,**c**,**d**,**j**,**r**,**s**,**y**) and different cell lines of non-small cell lung cancer. The most promising compound **5a** showed high activity on almost all cancer cell lines with mean growth—51.01%, where the most sensitive was melanoma cell line MDA-MB-435 with GP = −31.02%. The compound **5a** also possessed high activity on a number of leukemia and colon cancer cell lines (Table 1).

Additionally, in-vitro screening of antiproliferative activity of some the most promising and interesting compounds (**4c**, **4x**, **4y**, **5a**) towards several cancer cell lines (human acute T-cell leukemia cells of Jurkat, human leukemia, human colon carcinoma HCT116, human liver HepG2 and human ovarian cancer Skov3) was performed by the MTT assay. Tested compounds were added to cultured cells in different final concentrations (0–50 μM) and the cells were treated for 72 h. Doxorubicin

(0–10 μM) was used as a reference (positive control) drug. The obtained results were expressed as IC_{50} and presented in Figure 4.

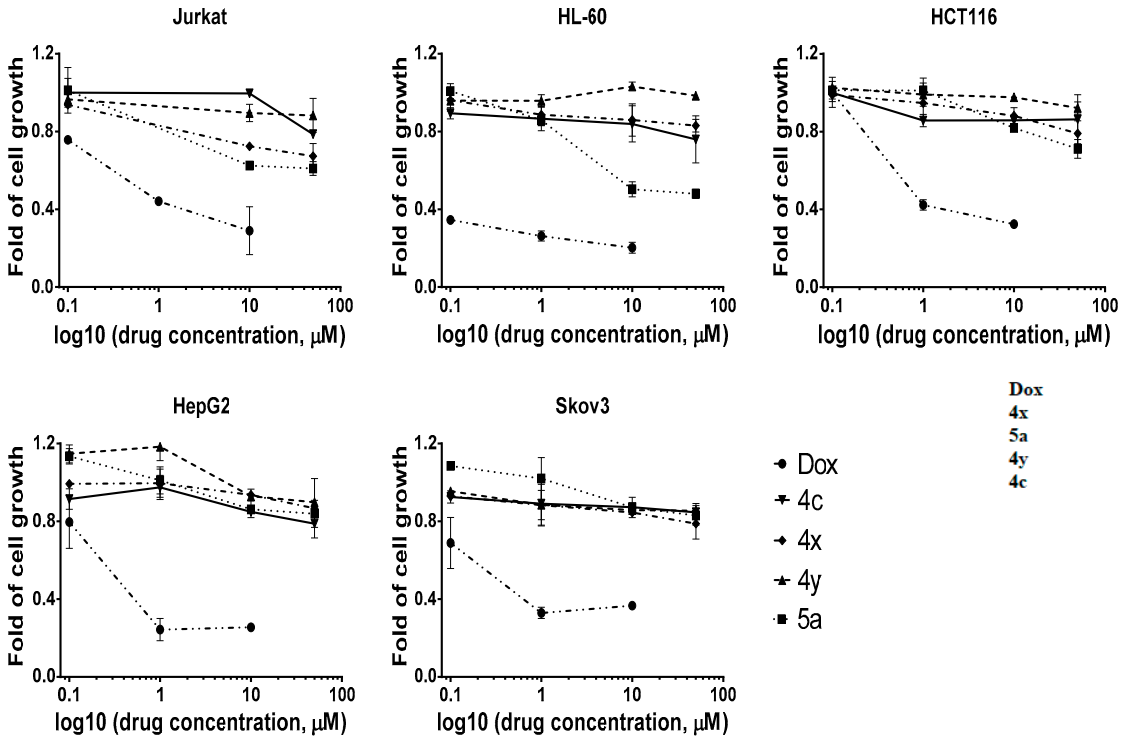


Figure 4. Cytotoxicity of thienopyrimidine derivatives towards human acute T-cell leukemia cells of Jurkat, human leukemia HL-60, human colon carcinoma HCT116, human liver HepG2 and human ovarian cancer Skov3 cell lines. After a total experimental time (72 h), cell vitality was detected by the MTT assay.

The synthesized thienopyrimidine **4x** displayed slight activity in vitro when screened against human leukemia cells of HL-60 line (IC_{50} = 10.2 ± 0.20 μM). Still, doxorubicin—"gold standard" in the chemotherapy—had more prominent effect (IC_{50} = 1.08 ± 0.12 μM, Figure 4). Unfortunately, the rest of the tested compounds **4c**,**x**,**y**, **5a** were not active against chosen cell lines at the concentration up to 50 μM. However, the activity of doxorubicin was higher than the activity of thienopyrimidines.

Based on the obtained results, SAR (Structure-Activity Relationship) could be proposed. In Figure 5, the main directions of the structure change that lead to an increase in activity are shown. It was observed that the most active compounds were found among thieno[2,3-*d*]pyrimidines. On the same cell lines, thieno[2,3-*d*]pyrimidines were more potent than the corresponding isomeric thieno[3,2-*d*]pyrimidines.

Moreover, in particular, an increase in bioactivity was characteristic when the phenyl substituent was changed to the annealed cyclohexyl and the introduction of the arylethylamine substituent in position 2 of the pyrimidine cycle was performed. This is shown by example of compounds **4**. Moreover, variation of the substituents in the thiophene core showed that compounds with cyclohexyl moiety are mostly more active, and by contrast, phenyl derivatives showed lower activity.

Utility of different amines in the synthetic protocol for the thienopyrimidine derivatives allowed us to evaluate the dependence between structure of the pyrimidine side chain and anticancer activity. It was found that the most active compound among thieno[2,3-*d*]pyrimidine-4(3*H*)-one is 2-(benzylamino)-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one **5a**, which possesses cytotoxic activity on the melanoma cell line MDA-MB-435 (GP = −31.02%). In addition, it should be noted that any

hindering of free rotation of the benzene fragment due to hydrogen bonds (Figure 6A) or attachment of the additional ethylene (Figure 6B) bridge lead to loss of activity.

As a result of the research, a number of compounds have been found that have shown good antitumor activity and patterns of structure–activity that are important for further optimization of the structure and the creation of more selective and active anticancer agents.

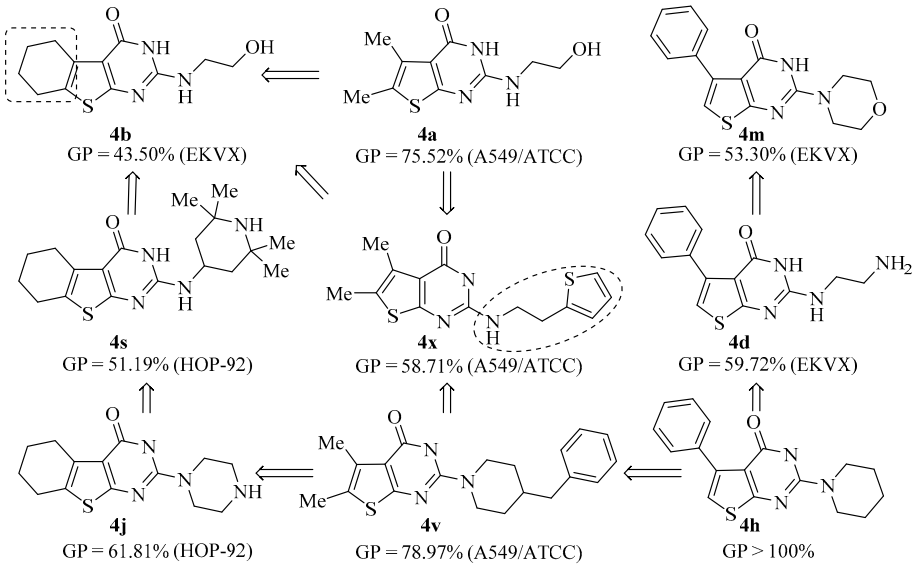


Figure 5. Structure–activity relationship among thieno[2,3-*d*]pyrimidines based on GP (Growth Percent) towards non-small cell lung cancer cell lines.

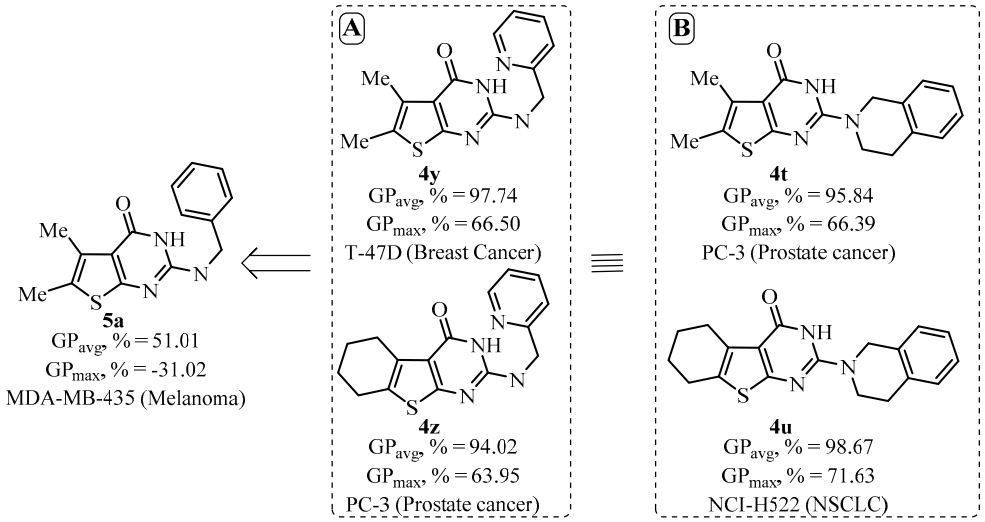


Figure 6. Structure–activity relationship among thieno[2,3-*d*]pyrimidines; influence of hindering of free rotation in the residues bearing pyrimidine ring. (**A**) Thieno[2,3-*d*]pyrimidines with pyridin-2-ylmethanamine moiety. (**B**) Thieno[2,3-*d*]pyrimidines with isoquinoline moiety.

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