Bioorganic Chemistry

ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2019. Vol. 35. N 2. P 321–330
doi: http://dx.doi.org/10.7124/bc.000A0F

UDC 547.859; 615.27

Anticancer activity evaluation of thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines and thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine derivatives

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Aim. In vitro evaluation of anticancer activity of synthesized thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines and thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines. **Methods.** Organic synthesis, *in vitro* cytotoxicity assay, MTT assay, spectrophotometry, statistical analysis. **Results.** The isomeric thienotriazolopyrimidines synthesized were tested for their anticancer activity in the NCI-60 cancer cell line panel, a group of 60 human cancer cell lines. The selective influence of 5-oxo-4,5,6,7,8,9-hexahydrobenzo[4,5]thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine-3-carboxamide on melanoma cell line SK-MEL-5 with (GP = -31,50%) was observed. Two compounds possessed a significant activity on CNS and breast cancer cells.**Conclusions.** Several thienotriazolopyrimidines were found to possess antitumor activity with a selective effect on a single cell line. These results are interesting for further structure optimization to increase selectivity and anticancer activity of fused pyrimidines.

K e y w o r d s: thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines, thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines, thienotriazolopyrimidines, anticancer activity, fused pyrimidine

Introduction

Thieno-fused five- and six-membered nitrogen containing heterocycles [1] play an important role in medical and pharmaceutical chemistry. One of the reasons is that several of fused cores are bioisosters to the natural purine bases. Noteworthy, the implementation of bioisosteric replacement strategy could be the basis for successful design of drug rational structure [2, 3]. In this regard, thienopyrimidines continue to attract considerable attention as the privileged scaffolds and the number of such core compounds, which exhibited various

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biological activities, gradually increases [4–6]. On the other hand, fused triazolopyrimidines also were found to be biologically active [7–9]. Moreover, we previously synthesized several 1,2,3-triazoles which were found to possess significant anticancer activity [10, 11]. Recently, we have developed facile synthetic methods for the versatile synthesis of 1,2,3-triazole and thieno-fused compounds, where new efficient and mild procedures from readily available reagents can be employed [12–26]. Those protocols could be successfully used for the preparation of fused heterocyclic compounds with both thiophene and 1,2,3-triazole rings. Based on such combination an extended structure-activity investigation focusing on drug-like properties should be performed.

The present work is devoted to the evaluation of anticancer activity of substituted thienotriazolopyrimidines synthesized at room temperature in a short time via domino- 1,3-dipolar cyclocondensation [16, 21, 12] that meets the energy-saving and environmentally friendly philosophical concepts of 'green chemistry'. The point of the research was to perform *in vitro* anticancer activity assay of the fused substituted thienotriazolopyrimidines.

Materials and Methods

Synthesis:

The thienotriazolopyrimidine derivatives were designed as promising anticancer agents and obtained as we described earlier [16, 21, 12].

Materials for MTT assay:

The 10 mM stock solution of thienotriazolopyrimidine derivatives was prepared in dimethyl sulfoxide (DMSO, Sigma–Aldrich, USA), and additionally dissolved in the culture medium prior to addition to the cell culture. *In vitro* screening of the anti-proliferative activity of the synthesized compounds and doxorubicin towards tumor cell was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test (99.5 % pure, Sigma-Aldrich, St. Louis, USA). The DMEM cell culture medium was obtained from Sigma–Aldrich, USA; RPMI-1640 – from PPA, Austria. Fetal bovine serum was obtained from Biowest, France. Doxorubicin was obtained from Actavis, Romania.

Cell cultures for MTT assay:

Human acute T-cell leukemia cells of Jurkat line were obtained from a Collection at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiology (Kyiv, Ukraine). Human myeloid leukemia HL-60 and human ovarian carcinoma cells of Skov3 line were from a Collection of the Institute of Cancer Research at Vienna Medical University (Vienna, Austria). Human hepatocarcinoma cells of HepG2 line and human colon carcinoma cells of HCT116 line were from a Collection at the Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine (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.

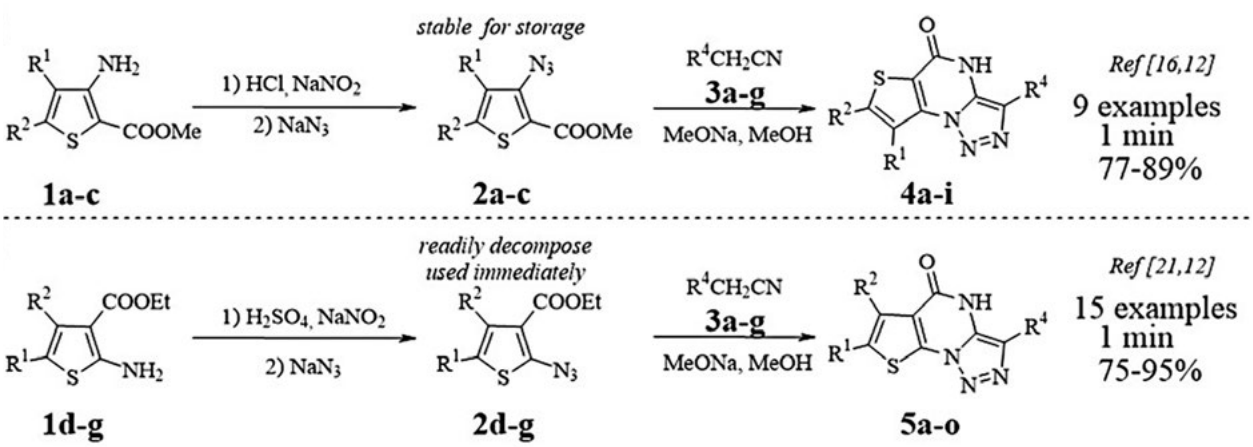
Anticancer assay via NCI protocol:

According to the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda, a primary anticancer assay was per-

formed within nine cancer types at approximately 60 human tumor cell lines panel. 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. Growth percent of 100 corresponds to growth seen in untreated cells. Growth percent of 0 indicates absence of net growth over the course of the assay (i.e. equal to the number of cells at time zero). Growth percent of -100 results when all cells are killed.

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 carried out by the MTT test [25]. Tumor cells were seeded for 24 hours in 96-well microtiter plates at a concentration of 5,000 substrate-dependent cells/well or 10,000 suspension cells/well (100 μL/well), after that cells were incubated for 72 hours with various additions of the synthesized compounds (0-50 μM). MTT, converted to dark violet, water insoluble MTT formazan by the mitochondrial dehydrogenases, was used to determine viable cells according to the Sigma-Aldrich protocol. Absorbance Reader BioTek ELx800 (BioTek Instruments, Inc., Winooski, VT, USA) was used for reaction results measurement.



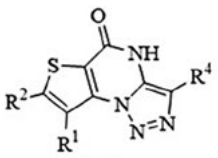
1,2: R^1=R^2=H(**a**), R^1=H, R^2=Ph (**b**), R^1+R^2=-$(CH_2)_4$- (**c**, **d**), R^1=R^2=Me (**e**), R^1=Me, R^2=C(O)Me (**f**), R^1=Me, R^2=COOEt (**g**), **3**: R^3= benzo[d]thiazol-2-yl (**a**), $C(O)NH_2$ (**b**), 1-Methylpyrrol-2-yl (**c**), CN (**d**), C(O)NHMe (**e**), 4-phenylthiazol -2yl (**f**), 1-amino-2,2-dicyanovinyl (**g**).

Scheme 1. Synthesis of thienotriazolopyrimidine derivatives.

Statistical analysis:

All data are presented as the mean (M) ± standard deviation (SD), n = 4. 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's multiple comparisons test.

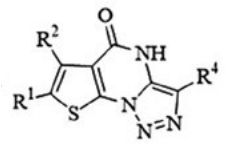
Table 1. **Anticancer screeening data of thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines 4a-i used at the concentration of 10-5 M**



№	Substituents	Mean growth, %	Range of growth, %	The most sensitive cell lines	Growth of the most sensitive cell lines, %
4a	R^1=R^2=H, R^4=Bth*	76.20	34.56 to 96.77	MDA-MB-468 (Breast Cancer) DU-145 (Prostate Cancer) NCI-H460 (Non-Small Cell Lung Cancer) NCI-H23 (Non-Small Cell Lung Cancer) RXF 393 (Renal Cancer) CCRF-CEM (Leukemia) HCT-116 (Colon Cancer) OVCAR-3 65.40 (Ovarian Cancer) A498 (Renal Cancer) MALME-3M (Melanoma)	31.56 47.35 52.21 53.95 56.12 61.46 63.90 65.40 65.57 65.67
4b	R^1=H, R^2=Ph, R^4=C(O)NH_2	93.18	58.67 to 111.03	SNB-75 (CNS Cancer) T-47D (Breast Cancer) UO-31 (Renal Cancer) NCI-H522 (Non-Small Cell Lung Cancer)	58.67 70.19 72.31 77.72
4c	R^1+R^2=-$(CH_2)_4$-, R^4=C(O)NH_2	101.11	85.43 to 116.57	UO-31 (Renal Cancer)	85.43
4d	R^1=H, R^2=Ph, R^4= C(NH)OMe	99.69	67.89 to 128.83	UO-31 (Renal Cancer)	67.89
4e	R^1=R^2=H, R^4=MP*	95.86	75.44 to 141.86	UO-31 (Renal Cancer) SNB-75 (CNS Cancer) NCI-H226 (Non-Small Cell Lung Cancer)	75.44 78.93 79.92
4f	R^1+R^2=-$(CH_2)_4$-, R^4=MP*	98.33	79.53 to 119.14	UO-31 (Renal Cancer)	79.53
4g	R^1= R^2=H, R^4=CN	100.59	88.26 to 115.89	SNB-75 (CNS Cancer)	88.26
4h	R^1+R^2=-$(CH_2)_4$-, R^4=CN	100.71	82.30 to 124.56	HOP-92 (Non-Small Cell Lung Cancer)	82.30
4i	R^1= R^2=H, R^4= ADCV*	99.70	79.63 to 128.78	UO-31 (Renal cancer) IGROV1 (Ovarian Cancer)	79.63 80.67

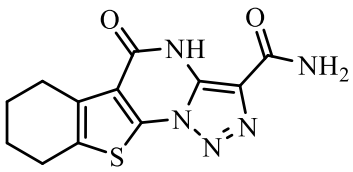
* MP = C(O)-1-Methylpyrrol-2-yl, Bth = Benzo[d]thiazol-2-yl, ADCV = 1-amino-2,2-dicyanovinyl.

Table 2. **Anticancer screening data of thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines 5a-o used at the concentration of 10-5 M**

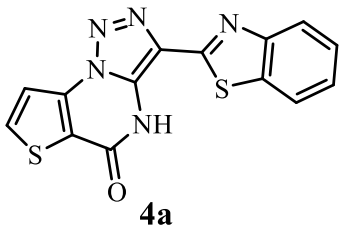


№	Substituents	Mean growth, %	Range of growth, %	The most sensitive cell lines	Growth of the most sensitive cell lines, %
5a	R^1+R^2=-$(CH_2)_4$-, R^4=C(O)NH_2	100.12	-31.50 to 120.43	SK-MEL-5 (Melanoma) CCRF-CEM (Leukemia) UO-31 (Renal Cancer)	-31.50 84.25 84.36
5b	R^1+R^2=-$(CH_2)_4$-, R^4=C(O)NHMe	98.54	78.24 to 109.10	CCRF-CEM (Leukemia)	78.24
5c	R^1=R^2=Me, R^4=C(O)NHMe	99.27	75.61 to 114.83	UO-31 (Renal Cancer)	75.61
5d	R^1+R^2=-$(CH_2)_4$-, R^4= C(NH)OMe	97.00	76.48 to 113.53	NCI-H522 (Non-Small Cell Lung Cancer)	76.48
5e	R^1=R^2=Me, R^4=CN	96.66	75.16 to 107.43	SNB-75 (CNS Cancer) HOP-92 (Non-Small Cell Lung Cancer) NCI-H522 (Non-Small Cell Lung Cancer)	75.16 76.61 78.12
5f	R^1=Me, R^2=C(O) Me, R^4=CN	99.57	83.77 to 112.08	UO-31 (Renal Cancer)	83.77
5g	R^1+R^2=-$(CH_2)_4$-, R^4= Ph	99.49	76.70 to 123.44	UO-31 (Renal Cancer)	76.70
5h	R^1=R^2=Me, R^4=Ph	100.33	80.89 to 109.93	UO-31 (Renal Cancer)	80.89
5i	R^1+R^2=-$(CH_2)_4$-, R^4=PTAz*	99.77	81.42 to 116.42	UO-31 (Renal Cancer)	81.42
5j	R^1=R^2=Me, R^4= PTAz*	97.20	76.17 to 144.63	UO-31 (Renal Cancer)	76.17
5k	R^1=Me, R^2=C(O) Me, R^4= PTAz*	98.03	75.46 to 130.52	UO-31 (Renal Cancer) T-47D (Breast Cancer)	75.46 79.86
5l	R^1=Me, R^2=COOEt, R^4= PTAz*	96.34	76.72 to 128.19	UO-31 (Renal Cancer)	76.72
5m	R^1=R^2=Me, R^4=Bth*	95.78	73.74 to 140.28	UO-31 (Renal Cancer) PC-3 (Prostate Cancer) MCF7 (Breast Cancer)	73.74 78.71 79.63
5n	R^1+R^2=-$(CH_2)_4$-, R^4= ADCV*	102.11	84.22 to 117.54	UO-31 (Renal cancer)	84.22
5o	R^1=R^2=Me, R^4= ADCV*	100.79	75.70 to 123.45	UO-31 (Renal cancer)	75.70

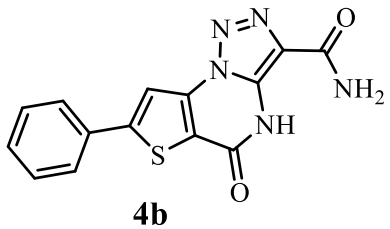
* -PTAz = 4-phenylthiazol -2yl, Bth = Benzo[d]thiazol-2-yl, ADCV = 1-amino-2,2-dicyanovinyl.



5a
GP, % = -31.50
SK-MEL-5 (Melanoma)



4a
GP, % = 31.56
MDA-MB-468 (Breast Cancer)



4b
GP, % = 58.67
SNB-75 (CNS Cancer)

Fig. 1. The most active compounds among studied thieno [2,3- and 3,2-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine-5(4*H*)-one

P-value of <0.05 was considered as statistically significant.

Results and Discussion

Chemistry

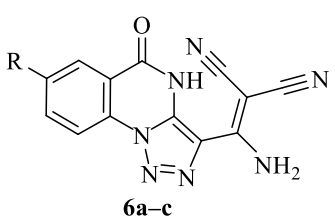
The compounds presented in the article were obtained using simple and convenient synthetic protocols (Scheme 1). The 3-aminothiophenes **1a-c** and Gewald thiophenes **1d-g** were used as starting materials in the synthesis of isomeric thienotriazolopyrimidine scaffolds **4** and **5**.

Starting alkyl amino-thiophene-carboxylates **1a-g** were converted into corresponding azides **2a-g,** which were found to be reactive in the base catalyzed cycloaddition with activated methylene compounds providing thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines **4** (Table 1) and thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines **5** (Table 2) with high yields according to previously reported synthetic protocols [16,21,12] (Scheme 1).

Evaluation of anticancer activity in vitro

The synthesized 24 examples of thienotriazolopyrimidines (**4a-i, 5a-o**) were submitted and evaluated at the single concentration of 10^{-5} M towards a panel of approximately sixty cancer cell lines. The human tumor cell lines were derived from nine different cancer types: leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostatev and breast cancers. Primary anticancer assays were performed according to the US NCI protocol (http://dtp.nci.nih.gov), which was described elsewhere [28-31]. The results for each compound are reported as the percent growth (GP) (Table 1) for thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*] pyrimidines and (Table 2) for thieno[2,3-*e*] [1,2,3]triazolo[1,5-*a*]pyrimidines. Range of growth (%) shows the lowest and the highest growth that was found among different cancer cell lines.

In comparison to previously synthesized thienopyrimidines [6] isomeric fused thieno[3,2-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines and thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines displayed slight or low activity in the *in vitro* screen on tested cell lines (Table 1, 2).



6a–c

6: R = H (**a**), R = Cl (**b**), R = Br (**c**)

Fig. 2. General structure of [1,2,3] triazolo[1,5-*a*] quinazolines **6a–c.**

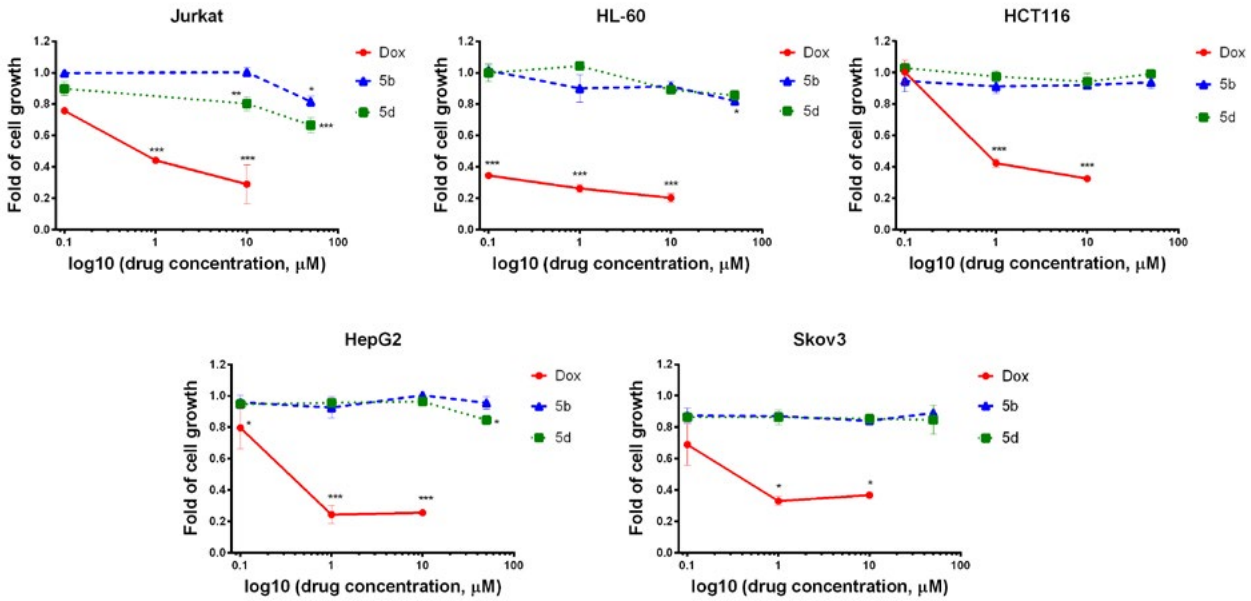


Fig. 3. Cytotoxicity of thienotriazolopyrimidines derivatives towards human acute T-cell leukemia cells of Jurkat line, human leukemia HL-60, human colon carcinoma HCT116, human liver HepG2 and human ovarian cancer Skov3 cell lines. After total time of the experiment (72 h), cell vitality was detected by the MTT assay.

Most of compounds possess activity against Renal Cancer UO-31 cell line. However, selective influence of compound **5a** on single melanoma cell line was observed, the compound was highly active on SK-MEL-5 cell line (GP = -31.50 %) (Table 2). Two other compounds **4a** and **4b** were found to be moderately active on MDA-MB-468 Breast Cancer cell line (GP = 31.56 %) and SNB-75 CNS Cancer cell line (GP = 58.67 %) respectively (Table 1). The most active compounds are presented in Figure 1.

Moreover, it should be noted that bioisosteric to thieno[2,3- and 3,2-*e*][1,2,3] triazolo[1,5-*a*]pyrimidine-5(4*H*)-ones **4i, 5n,o** [1,2,3]triazolo[1,5-*a*]quinazolines **6a-c** (Fig. 2) [12] were not selected by NCI for *in vitro* anticancer screening. The single selected and tested by NCI 2-(amino(5-amino-[1,2,3] triazolo[1,5-*a*]quinazolin-3-yl)methylene) malononitrile **6a** was slightly active against Renal Cancer UO-31 cell line (GP = 81.85 %) still with low mean growth of 100.20 %. Thus, thieno- fused [1,2,3]triazolo[1,5-*a*]pyrimidines were found to be more active in comparison to corresponding "aryl-fused [1,2,3] triazolo[1,5-*a*]pyrimidines" – [1,2,3] triazolo[1,5-*a*]quinazolines **6a–c**.

Additionally, *in vitro* screening of anti-proliferative activity of some compounds (**5b, 5d**) in different final concentrations (0-50 μM) towards several cancer cell lines (human acute T-cell leukemia cells of Jurkat line, human colon carcinoma HCT116, human liver HepG2 and human ovarian Skov3) was performed by the MTT assay. However, the activity of doxorubicin was higher than the cytotoxicity of synthesized thienotriazolopyrimidines **5b, 5d**

at the concentration up to 50 μM against chosen cell lines (Figure 3).

Conclusion

In the present article, *in vitro* anticancer activity of the isomeric thienotriazolopyrimidines was evaluated. These preliminary results allowed identifying the most active compounds: 24 of the synthesized compounds were tested and two of them **4a, 4b** displayed moderate antitumor activity against CNS and breast cancers cell lines. Moreover, the compound **5a** can be defined as a prospective antitumor agent with the value of GP = -31.50 % on SK-MEL-5 melanoma cell line, the compound has selective influence being active only against a single cell line. The obtained results of antitumor activity of such derivatives are interesting with the hope to get more selective and active anticancer agents among fused pyrimidines and prove the necessity of further investigation.

Acknowledgments

We are grateful to Dr. V.L. Narayanan from Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, USA, for *in vitro* evaluation of anticancer activity.

Funding

The authors are grateful to the Ministry of Education and Science of Ukraine for financial support of this project (Grant No 0116U008067, No 0118U003610).

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Вивчення протиракової активності похідних тієно[3,2-*e*][1,2,3]триазоло[1,5-*a*]піримідинів і тієно [2,3-*e*][1,2,3]триазоло[1,5-*a*]піримідину

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Мета. вивчення протиракової активності тієно[3,2-*e*][1,2,3]триазоло[1,5-*a*]піримідинів і тієно [2,3-*e*][1,2,3]триазоло[1,5-*a*]піримідинів. **Методи.** Органічний синтез, аналіз цитотоксичності *in vitro*, МТТ-аналіз, спектрофотометрія, статистичний аналіз. **Результати.** Виявлено селективний вплив 5-оксо-4,5,6,7,8,9-гексагідробензо [4,5]тієно[3,2-*e*][1,2,3]триазоло[1,5-*a*] піримідин-3-карбоксаміду на лінію клітин меланоми SK-MEL-5 з (GP = -31,50 %). Більшість синтезованих сполук показали помірну протиракову активність. Дві з них мали високу активність щодо раку ЦНС і раку молочної залози. **Висновки.** Виявлено низку тієнотриазолопіримідинів, що володіють протипухлинною активністю і, зокрема, селективно діють лише на одну клітинну лінію. Такі результати є цікавими для подальшого дослідження для отримання більш селективних і активних протипухлинних засобів серед конденсованих піримідинів.

Ключові слова: тієно[3,2-*e*][1,2,3]триазоло[1,5-*a*] піримідини, тієно[2,3-*e*][1,2,3]триазоло [1,5-*a*]піримідини, тієнотриазолопіримідини, протиракова активність, конденсовані піримідини

Оценка противораковой активности производных тиено[3,2-*e*][1,2,3]триазоло[1,5-*a*] пиримидинов и тиено[2,3-*e*][1,2,3] триазоло[1,5-*a*]пиримидинов

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Цель. изучить противоопухолевую активность тиено[3,2-*e*][1,2,3]триазоло[1,5-*a*] пиримидинов и тиено[2,3-*e*][1,2,3]триазоло[1,5-*a*]пиримидинов. **Методы.** Органический синтез, анализ цитотоксичности *in vitro*, анализ МТТ, спектрофотометрия, статистический анализ. **Результаты.** Обнаружено избирательное влияние5-оксо-4,5,6,7,8,9-гексагидробензо[4,5]тиено[3,2-*e*][1,2,3]триазоло[1,5-*a*]пиримидин-3-карбоксамида только на линию меланомы SK-MEL-5 с (GP = -31,50%). Большинство синтезированных соединений проявили слабую противоопухолевую активность. Двое из них обладали значительной активностью в отношении рака ЦНС и рака молочной железы. **Выводы.** обнаружено, что некоторые тиенотриазолопиримидины обладают противоопухолевой активностью и, в частности, оказывают избирательное влияние только на одну клеточную линию. Такие результаты представляют интерес для дальнейшего изучения, чтобы получить более селективные и активные противораковые агенты среди конденсированных пиримидинов.

Ключевые слова: тиено[3,2-*e*][1,2,3]триазоло[1,5-*a*]пиримидины, тиено[2,3-*e*][1,2,3] триазоло[1,5-*a*]пиримидины, тиенотриазолопиримидины, противораковая активность, конденсированные пиримидины

Received 26.08.2018