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SYNTHESIS, STRUCTURAL CHARACTERIZATION AND ANTITUMOR ACTIVITY OF NEW CHROMENO[4′,3′:4,5]THIOPYRANO[2,3-*d*]THIAZOLE DERIVATIVES

Mykhailo Hoidyk, Andriy Karkhut, Svyatoslav Polovkovych, Roman Lesyk

The development of heterocyclic compounds with significant biological activity remains a priority in modern medicinal chemistry. The use of cascade domino reactions, such as Knoevenagel condensation combined with hetero-Diels-Alder cyclization, enables the efficient construction of complex structures with potential anticancer properties.

The aim of the study. *To synthesize a series of thiopyrano[2,3-d]thiazole derivatives via a cascade Knoevenagel-hetero-Diels-Alder reaction followed by N3-alkylation and evaluate their in vitro antitumor activity in the NCI-60 human cancer cell line panel.*

Materials and methods. *Structural identification of the compounds was carried out using NMR spectroscopy in DMSO-d_6 with tetramethylsilane (TMS) as the internal standard, and LC-MS analysis with an APCI mass-selective detector. Biological activity was assessed using the NCI-60 screening program, which includes a panel of 60 human cancer cell lines of various origins. Key parameters such as growth inhibition (GI_{50}), lethal concentration (LC_{50}), and cytotoxicity at micromolar concentrations were determined.*

Results. *A series of thiopyrano[2,3-d]thiazole derivatives were synthesized through a two-step domino Knoevenagel condensation and intramolecular hetero-Diels–Alder cyclization between 4-thioxo-2-oxothiazolidinone and O-alkylated salicylaldehyde derivatives bearing allylic or propargyl substituents. Subsequent N3-alkylation yielded compounds 3.1 (60.0%), 3.2 (67.0%), and 4 (58.0%). Introduction of a piperidine moiety enabled the synthesis of water-soluble methanesulfonate salt 5 (70.0%). Reaction with 2,5-(2-propynyloxy)benzaldehyde led to in situ aromatization and the formation of a stable compound 8. Four compounds were tested for anticancer activity. Compound 8 showed the highest efficacy, causing complete cell death in OVCAR-4 (Ovarian Cancer, LC_{50} = 29.5 μM) and strong growth inhibition in SR (Leukemia, GI_{50} = 0.676 μM), 786-0 (Renal Cancer, GI_{50} = 0.696 μM), A498 (Renal Cancer, GI_{50} = 0.528 μM), and BT-549 (Breast Cancer, GI_{50} = 0.666 μM) cells.*

Conclusions. *The proposed synthetic methodology enables efficient preparation of structurally diverse thiopyrano[2,3-d]thiazole derivatives in high yields. N3-alkylation and incorporation of a piperidine fragment allowed for the synthesis of a water-soluble methanesulfonate salt 5. Among the tested compounds, compound 8 exhibited the most promising cytotoxicity and selectivity towards several cancer cell lines, suggesting its potential as a lead compound for further preclinical development of novel anticancer agents*

Keywords: *anticancer activity, thiopyrano[2,3-d]thiazoles, Knoevenagel condensation, hetero-Diels–Alder reaction, domino reaction*

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1. Introduction

Malignant neoplasms continue to be one of the main problems of modern medicine, as they are one of the main causes of mortality in the world [1–4]. Oncological diseases constitute a complex and heterogeneous set of more than 200 pathologies, united under the generalized term “cancer”, which differ significantly in histogenesis, etiology, pathogenesis, clinical course, molecular characteristics and sensitivity to treatment. According to the Cancer Research Institute (2023), the most common forms of cancer in the world remain lung cancer, breast cancer, colorectal cancer, prostate cancer, bladder cancer and melanoma [5]. These types of malignant tumors are responsible for almost 50% of all cases of cancer diagnosis in the USA and European countries [5]. In the EU countries in 2018, approximately 1.6 million new cases of cancer were recorded among men and 1.4 million among women. During the same period, about 790,000 men and 620,000 women died from cancer [6]. Breast cancer had the highest incidence in the region, with over 523,000 new cases, making it the leading form of cancer among women, especially in the over-50 age group [5]. Colorectal cancer was the second most common cancer with 500,000 reported cases and 243,000 deaths, indicating the high lethality of the disease [7]. Lung cancer was the third most frequently diagnosed cancer (470,000 cases) but was the leading cause of death with 388,000 deaths in the EU [7]. Prostate cancer, with 450,000 new cases, is the fourth most common, and globally it kills about 360,000 men each year [7]. Skin melanoma remains particularly common among young adults aged 25–40 years, with 97,610 new cases reported in the US in 2023 and over 325,000 worldwide in 2020 according to GLOBOCAN [8–11].

Bladder cancer had an estimated 82,000 new cases in the US alone in 2023. This disease is characterized by a high risk of recurrence even after radical treatment, which necessitates the need for continuous monitoring of patients and the development of more effective regimens for the prevention of tumor recurrence [12]. Taken together, these data indicate an extremely high level of oncological morbidity and mortality, which necessitates the urgent need for further development of effective methods of prevention, early diagnosis and treatment of malignant neoplasms [13].

Thiazole derivatives and related structures are of special interest in the context of the search for new anticancer agents, given the proven effectiveness of the thiazole cycle as a structural basis for the creation of biologically active molecules. As of 2021, the list of FDA-approved drugs includes 18 drugs containing a thiazole cycle, as well as many experimental compounds with a similar fragment in the structure [14]. Among thiazole-containing heterocycles, a special place is occupied by 4-thiazolidinones [15–19], in particular 5-en-4-thiazolidinones, which form a well-studied class of compounds with a wide spectrum of biological activity [20–26]. At the same time, the extraordinary pharmacological diversity of this subgroup has become the basis for the classification of 5-ene-4-thiazolidinones as pan-assay interference compounds (PAINS) – i. e. compounds that exhibit non-specific activity against several biological targets [27, 28]. This is due to their polyfunctionality, limited selectivity, and the ability to act as Michael acceptors. In view of the above, thiopyrano[2,3-*d*]thiazole derivatives are considered as promising bioisosteric analogues of 5-ene-4-thiazolidinones, which are able to preserve the characteristic biological profile of the latter, but at the same time are devoid of the typical disadvantages associated with non-specific activity and reactivity inherent in Michael acceptors [29–33].

A few studies [34–37] have demonstrated the potential of quinoid derivatives of thiopyrano[2,3-*d*] thiazole as potential antitumor agents. Some members of this class have shown marked cytotoxic activity against colorectal cancer cells, inducing the accumulation of reactive oxygen species, cell cycle arrest in the S and G_2/M phases, inhibition of DNA biosynthesis, and activation of caspases 3/7, 8, 9, and 10, suggesting the initiation of apoptosis via the intrinsic and extrinsic pathways [34]. Other quinone-containing derivatives of thiopyranothiazoles have demonstrated cytotoxicity comparable to doxorubicin (IC_{50} = 0.60–5.98 μM) but have lower toxicity against normal cells and have not caused acute toxicity *in vivo*, which underlines their biocompatibility and therapeutic potential [35]. It has also been found that some molecules of this group selectively act on epidermoid and colorectal cells, and their activity depends on the status of p53 in the cells, suggesting a possible role of this protein in the mechanism of action [36]. Some fused analogues of thiopyranothiazoles have shown moderate selectivity for melanoma cells with low toxicity [31]. In the framework of studies [37–41], new thiazole derivatives were synthesized and characterized, which showed promising antitumor activity. The thiopyrano[2,3-*d*]thiazol-2-ones described in [39] demonstrated significant inhibition of proliferation of leukemia, lung, colon, CNS, melanoma, prostate and breast cancer cells with GI_{50} in the range of 0.37–0.67 μM. Further study [40] of seven chromeno[4',3':4,5]thiopyrano[2,3-*d*]thiazoles showed the ability of one of them to induce apoptosis in Jurkat T-leukemia cells and U251 glioblastoma cells by a mechanism dependent on reactive oxygen species and PARP-1 inhibition, while maintaining low toxicity to normal cells (IC_{50} > 100 μM). Previous works also described the synthesis of *rel*-(5*R*,6*S*,7*S*)-thiopyrano[2,3-*d*]thiazoles, among which agents with moderate cytotoxicity to HT-29 colorectal cancer cells, as well as antiexudative activity under conditions of the inflammatory process *in vivo* were identified [41].

In addition to the revealed antitumor activity, thiazole and thiopyrano[2,3-*d*]thiazole derivatives demonstrate a wide spectrum of biological activity, which is due to the high potential of their heterocyclic core as a pharmacophore. The structural rigidity, electron density and the possibility of variable functionalization of this system open up prospects for the search for new molecules with other types of pharmacological action. In particular, recently there has been growing interest in studying the anticonvulsant [42–44], antiparasitic [45–50] and antimicrobial [31, 51, 52] activities of these compounds, which is due to the need for new effective agents with an improved safety profile and selectivity. In this context, thiopyrano[2,3-*d*]thiazoles are considered a promising scaffold for the development of multifunctional drugs.

2. Planning (methodology) of the research

The study design was based on the principles of synthesis and biological evaluation of novel thiopyrano[2,3-*d*]thiazoles as promising anticancer (Fig. 1).

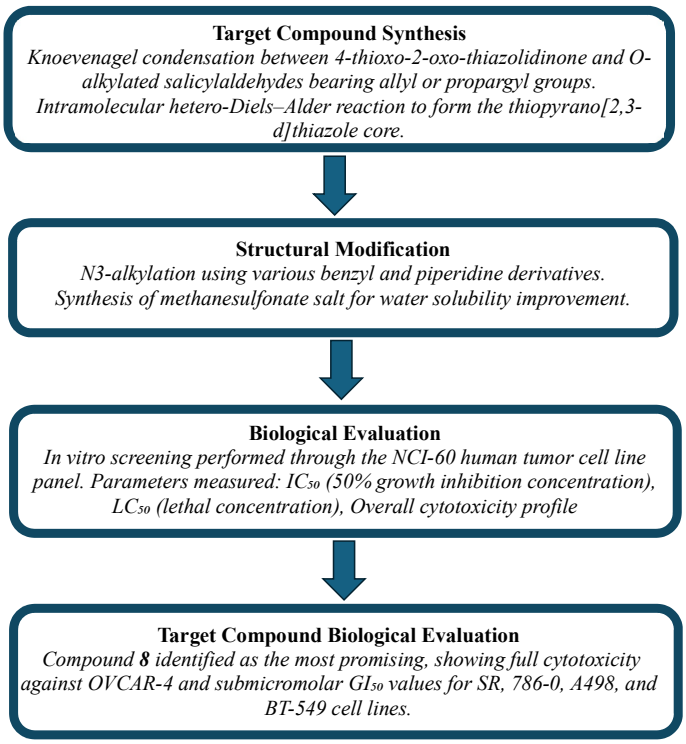

Fig. 1. Stepwise strategy for the rational development and screening of novel Thiopyrano[2,3-*d*]thiazoles

The methodological approach relied on the application of cascade domino reactions, structural variation, and stepwise bioanalysis. The synthetic part involved a two-step Knoevenagel condensation followed by intramolecular heterocyclization *via* the *hetero*-Diels–Alder mechanism to efficiently construct the heterocyclic core. To optimize drug-like properties, N3-alkylation was performed, along with the synthesis of a water-soluble methanesulfonate salt. Biological activity was assessed using the NCI-60 screening program, which includes 60 human cancer cell lines of various origins. Key evaluation parameters included GI_{50} (growth inhibition), LC_{50} (lethal concentration), and overall cytotoxicity. This approach enabled the identification of compound **8** as a promising candidate for further investigation.

3. Materials and methods

All reagents and solvents were purchased from commercial suppliers and were used directly without further purification. NMR spectra were determined with Varian Unity Plus 400 (400 MHz) and Bruker 170 Avance 500 (500 MHz) spectrometers, in DMSO-d6 using tetramethylsilane (TMS) as an internal standard. Melting points were measured on a Kofler hot-stage and are uncorrected. LC-MS was performed using a system with an Agilent 1100 Series HPLC equipped with diode-array detector and Agilent LC\MSD SL mass-selective detector using chemical ionization at atmospheric pressure (APCI).

General procedure for the synthesis of 3.1, 3.2, 4. (5aS,11bS)-3-(2-Chlorobenzyl)-3,5a,6,11b-tetrahydro-2H,5H-chromeno[4',3':4,5]thiopyrano[2,3-d]thiazol-2-one (3.1). To a solution of potassium hydroxide (73 mg, 0.0013 mol) in ethanol (8 ml) compound **2a** (0.360 g, 0.0013 mol) was added and kept at room temperature with stirring for 15 min to form the corresponding potassium salt. Then 2-chlorobenzyl chloride **1.1** (0.242 g, 0.0015 mol) was added in one portion and the reaction mixture was refluxed for 2.5 h. After cooling the product was precipitated with water, filtered, dried and recrystallized from DMF/ethanol (1:2). Yellow powder, yield 0.313 g (60%), m.p. 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 7.49 (*t*, *J* = 7.5 Hz, 1H, Ar), 7.38–7.29 (*m*, 2H, Ar), 7.25 (*d*, *J* = 7.3 Hz, 1H, Ar), 7.23–7.16 (*m*, 2H, Ar), 7.00 (*t*, *J* = 7.8 Hz, 1H, Ar), 6.94 (*d*, *J* = 8.2 Hz, 1H, Ar), 6.83 (*d*, *J* = 8.1 Hz, 1H, Ar), 4.87 (*s*, 2H, CH_2 benzyl), 4.29 (*d*, *J* = 10.4 Hz, 1H, CH-11b), 4.15–4.05 (*m*, 2H, 6-CH_2), 3.47 (*d*, *J* = 13.5 Hz, 1H, 5-CH_2), 3.23 (*dd*, *J* = 13.5, 5.7 Hz, 1H, 5-CH_2), 2.75 (br.s, 1H, 5a-CH). m/z = 402 ([M+H]$^+$).

(5aS,11bS)-3-(3-Chlorobenzyl)-3,5a,6,11b-tetrahydro-2H,5H-chromeno[4',3':4,5]thiopyrano[2,3-d]thiazol-2-one (3.2). The reaction was carried out similarly to the synthesis of compound **3.1**, using 3-chlorobenzyl chloride **1.2** (0.0015 mol). Yellow powder, yield 0.350 g (67%), m.p. 153–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 7.42–7.32 (*m*, 3H, Ar), 7.29 (*s*, 1H, Ar), 7.26–7.12 (*m*, 4H, Ar), 6.93 (*t*, *J* = 7.4 Hz, 1H, Ar), 6.82 (*d*, *J* = 8.1 Hz, 1H, Ar), 4.81 (s, 2H, CH_2 benzyl), 4.30 (*d*, *J* = 10.4 Hz, 1H, CH-11b), 4.13–3.97 (*m*, 2H, 6-CH_2), 3.49 (*d*, *J* = 13.0 Hz, 1H, 5-CH_2), 3.25 (*dd*, *J* = 12.6, 4.1 Hz, 1H, 5-CH_2), 2.74 (br.s, 1H, 5a-CH). m/z = 402 ([M+H]$^+$).

(5aS,11bS)-8,10-Dibromo-3-(2-(piperidin-1-yl)ethyl)-3,5a,6,11b-tetrahydro-2H,5H-chromeno[4',3':4,5]thiopyrano[2,3-d]thiazol-2-one (4). To a solution of potassium hydroxide (0.167 g, 0.00298 mol) in ethanol (10 ml) was added compound **2b** (0.650 g, 0.00149 mol) and kept under stirring at room temperature for 15 minutes. Then 1-(2-chloroethyl)piperidine hydrochloride **1.3** (0.534 g, 0.0029 mol) was added in one portion. The reaction mixture was refluxed for 2.5 hours. After completion of the reaction and cooling, the product was filtered, dried and recrystallized from acetonitrile. Yellow powder, yield 0.472 g (58%), m.p. 144–146 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 7.72 (*d*, *J* = 2.2 Hz, 1H, Ar), 7.45 (*d*, *J* = 2.2 Hz, 1H, Ar), 4.49 (*d*, *J* = 9.8 Hz, 1H, CH-11b), 4.27 (*t*, *J* = 10.3 Hz, 1H, 6-CH_2), 4.14 (*d*, *J* = 3.5 Hz, 1H, 6-CH_2), 3.69–3.57 (*m*, 2H, -CH_2-CH_2-pip.), 3.51 (*d*, *J* = 12.9 Hz, 1H, 5-CH_2), 3.30–3.22 (*m*, 1H, 5-CH_2), 2.79 (br.s, 1H, 5a-CH), 2.43 (*t*, *J* = 6.0 Hz, 2H, -CH_2-CH_2-pip.), 2.34 (br.s, 4H, 2CH_2 pip.), 1.44 (br.s, 4H, 2CH_2 pip.), 1.34 (br.s, 2H, CH_2 pip.). m/z = 547.0 ([M+H]$^+$).

1-(2-((5aS,11bS)-8,10-Dibromo-2-oxo-5a,11b-dihydro-2H,5H-chromeno[4',3':4,5]thiopyrano[2,3-d]thiazol-3(6H)-yl)ethyl)piperidin-1-ium methanesulfonate (5). To a solution of compound **4** (0.500 g, 0.915 mmol) in tetrahydrofuran (3 ml) was added methanesulfonic acid (0.088 g, 0.915 mmol). The reaction mixture was heated to 40°C and stirred for 5 min. After cooling of the reaction mixture, the solvent was evaporated under vacuum, the resulting crystalline solid was mixed with diethyl ether, filtered, washed with diethyl ether, dried and used without further purification. Pink powder, yield 0.405 g (70%), m.p. 221–223 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 9.11 (*s*, 1H, N^+H), 7.74 (*d*, *J* = 2.1 Hz, 1H, Ar), 7.47 (*d*, *J* = 2.1 Hz, 1H, Ar), 4.49 (*d*, *J* = 10.6 Hz, 1H, CH-11b), 4.32 (*t*, *J* = 10.2 Hz, 1H, 6-CH_2), 4.16 (*d*, *J* = 4.3 Hz, 1H, 6-CH_2), 4.02 (*dt*, *J* = 15.0, 7.2 Hz, 1H, -CH_2-CH_2-pip.), 3.91 (*dt*, *J* = 15.0, 7.2 Hz, 1H, -CH_2-CH_2-pip.), 3.55 (*q*, *J* = 11.4, 10.4 Hz, 2H, -CH_2-CH_2-pip.), 3.35 (*dd*, *J* = 13.5, 5.7 Hz, 2H, pip.), 3.30–3.19 (*m*, 2H, 5-CH_2), 2.94 (*q*, *J* = 11.6 Hz, 2H, pip.), 2.83 (*s*, 1H, 5a-CH), 2.32 (*s*, 3H, CH_3 Ms), 1.82 (*d*, *J* = 11.6 Hz, 2H, pip.), 1.72–1.53 (*m*, 3H, pip.), 1.38 (*t*, *J* = 13.3 Hz, 1H, pip.). m/z = 547.0 ([M+H]$^+$).

9-(Prop-2-yn-1-yloxy)-2H,6H-chromeno[4',3':4,5]thiopyrano[2,3-d]thiazol-2-one (8). To a solution of 4-thioxo-2-oxothiazolidinone **1** (0.480 g, 0.0036 mol) in acetonitrile (30 mL) containing EDDA catalyst (0.059 g, 0.0004 mol) was added 2,4-bis(prop-2-yn-1-yloxy)benzaldehyde **6** (1.070 g, 0.005 mol). After stirring at room temperature for 15 min, the reaction mixture was refluxed for 2 h. After completion of the reaction and cooling, the precipitate was filtered, dried, and purified by recrystallization from DMF/ethanol (1:2). Yellow powder, yield 0.707 g (60%), m.p. 264–265 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.33 (*d*, *J* = 1.4 Hz, 1H, Ar), 8.03 (*dd*, *J* = 9.0, 1.6 Hz, 1H, Ar), 6.97 (*ddd*, *J* = 9.0, 2.6, 1.5 Hz, 1H, Ar), 6.82 (*dd*, *J* = 2.6, 1.6 Hz, 1H, -CH=), 5.12 (*s*, 2H,

-CH_2-), 4.97–4.91 (*m*, 2H, -CH_2-), 3.68 (*q*, *J* = 2.2 Hz, 1H, ≡C–H). m/z = 328.0 ([M+H]$^+$).

Antitumor activity studies were performed for the compounds **3.1**, **3.2**, **5**, **8** according to the NCI DTP (USA) standard protocol [53–57].

4. Results

Modern medicinal chemistry faces a number of challenges, including high resource intensity, multi-step, complexity of synthesis of drug candidates and limited possibility of their structural modification [58–60]. In response to these challenges, the scientific community is actively implementing energy-saving and environmentally friendly synthetic strategies based on the principles of green chemistry, click chemistry and accelerated molecular design [58, 60–62], these approaches form a powerful basis for the creation of multi-billion chemical spaces with high potential for medicinal applications. Multicomponent reactions [63–65], single-reactor synthesis [66, 67], cascade and tandem reactions [68, 69], as well as domino sequences [70, 71] are becoming increasingly widespread. Such reactions significantly reduce the number of steps, solvent consumption, and waste, while providing high chemical efficiency, synthetic flexibility, and structural diversity – key factors in the search for new biologically active compounds.

In our study, an efficient approach to the synthesis of new thiopyrano[2,3-*d*]thiazoles were implemented by a domino sequence of Knoevenagel condensation reactions and *hetero*-Diels-Alder cycloaddition. Within this approach, a series of target compounds was synthesized by a diastereoselective two-step domino reaction.

In the first step, 4-thioxo-2-thiazolidinone **1** was condensed with O-alkylated derivatives of salicylic aldehydes **1a,b** and **6**. The presence of an allylic or propargyl moiety in the *o*-position of aldehydes **1a,1b** (Fig. 2) and **6** (Fig. 3) allows their further intramolecular cyclization with the formation of a thiopyran ring in the second step. The reaction to prepare thiopyrano[2,3-*d*] thiazoles **2a,b** was carried out in acetonitrile using ethylenediamine diacetate (EDDA) as an effective base catalyst at reflux for 2 h (Fig. 2). Compound **2a** was obtained in 64% yield, while compound **2b** was obtained in 61.3% yield.

For further structural modification, N3-functionalization of derivatives **2a** and **2b** was carried out by alkylation with 2-chlorobenzyl chloride **1.1**, 3-chlorobenzyl chloride **1.2** and 1-(2-chloroethyl)piperidine hydrochloride **1.3**. The reactions were carried out in ethanol in the presence of KOH due to the preliminary formation of potassium salts of the starting thiopyrano[2,3-*d*]thiazoles **2a**, **2b**. As a result, the corresponding derivatives **3.1** (60.0%), **3.2** (67.0%) and **4** (58.0%) were synthesized. It is important to note that the introduction of the piperidine fragment allowed us to obtain a water-soluble methanesulfonate salt **5** with a yield of 70.0%, which was obtained by the action of methanesulfonic acid on **4** in tetrahydrofuran (Fig. 2).

During the interaction of 4-thioxo-2-oxothiazolidinone **1** with 2,5-(2-propynyloxy)benzaldehyde **6** in acetonitrile with the presence of EDDA as a catalyst, a stepwise process of formation of the thiopyrano[2,3-*d*] thiazole nucleus was followed by subsequent aromatization *in situ* (Fig. 3).

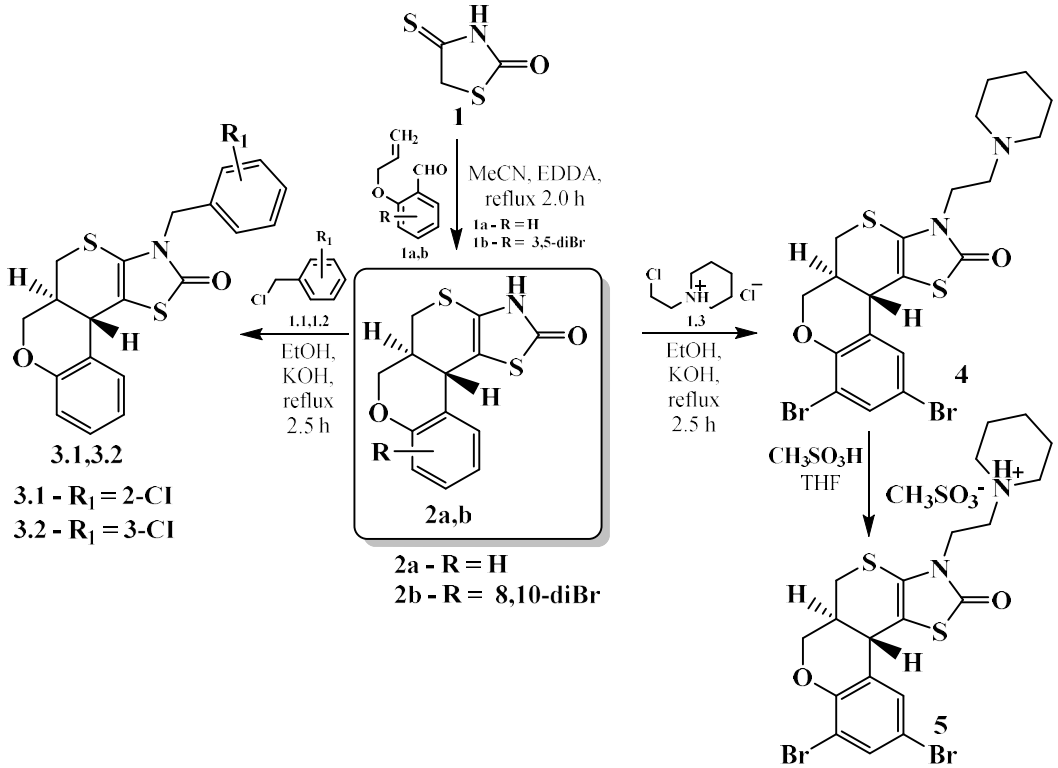

Fig. 2. Synthesis and N3-functionalization of thiopyrano[2,3-d]thiazole derivatives **2a, 2b, 3.1, 3.2, 4** and methanesulfonate salt **5**

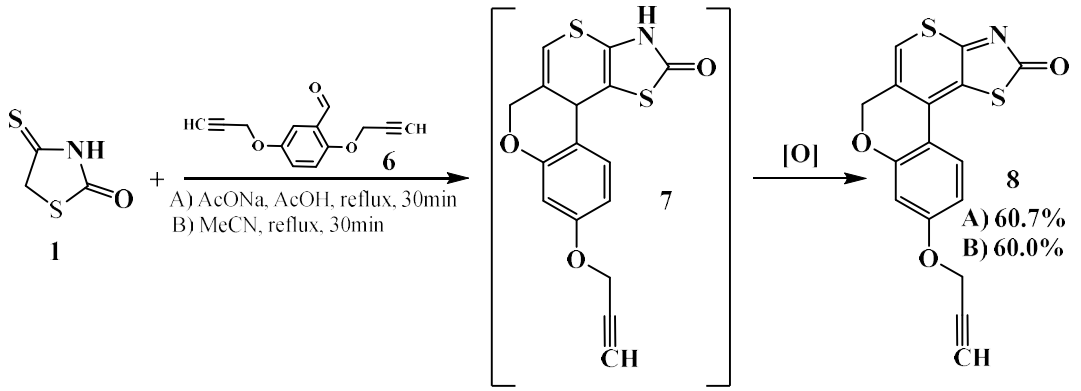

Fig. 3. Synthesis of compound **8** via Knoevenagel condensation and intramolecular hetero-Diels-Alder cyclization

Expected product **7** was not isolated, probably due to the high reactivity and instability of the specified structure in air, which led to its immediate oxidation to the more stable aromatic system **8** with 60.0% yield. The reaction can be carried out in boiling acetic acid with sodium acetate as a catalyst with almost the same yield (60.7%) of oxidized product **8**, compound **7** was also not isolated.

The obtained compounds were characterized using ^{1}H NMR spectroscopy and mass spectrometry, which allowed us to reliably confirm the formation of the target heterocyclic systems. In particular, the aromatization of the intermediate product **7** is confirmed by the absence of expected saturated thiopyran ring proton signals in the range of 3.8–4.5 ppm in the ^{1}H NMR spectrum of isolated product **8**. Instead, only one proton of unsaturated thiopyran ring at $\delta = 6.82$ ppm in the absence of short-range couplings (doubled doublet with $J = 2.6$ and 1.6 Hz) is observed. The mass spectrum of isolated product demonstrated an ion with m/z = 328.0 ([M+H]$^+$), which also corresponds to the oxidized aromatic structure **8**. The totality of the data obtained reliably verifies the course of *in situ* oxidation of the intermediate product with the formation of a stable aromatic heterocyclic structure. The configuration of Diels-Alder reaction products **2a,b** is also confirmed by ^{1}H NMR spectroscopy. The spin-spin coupling constant between the **5a** and **11b** protons is about 10 Hz and indicates *trans*-configuration with a dihedral angle close to 180°. It proves that under reaction conditions the subsequent cycloaddition occurs diastereoselectively with the formation of *exo*-products due to steric factors.

In order to determine the potential of the synthesized thiopyrano[2,3-*d*]thiazoles (**3.1**, **3.2**, **5**, **8**) as antitumor agents, a screening of biological activity was performed on a standard panel of 60 human cancer cell lines (NCI, USA) at a fixed concentration of 10^{-5} M (Table 1). The results obtained indicate a pronounced cytostatic and cytotoxic effect of some compounds, with varying degrees of selectivity and spectrum of activity.

Compound **3.1** showed moderate cytostatic activity against two breast cancer cell lines **HS 578T** and **T-47D**, where the growth inhibition was 59.45% and 53.31%, respectively. At the same time, the other 50 tested cell lines showed low sensitivity to this compound, which indicates a narrow spectrum of action and a possible specificity of the mechanism of action. The level of inhibition of the growth of **HS 578T** and **T-47D** cells for **3.2** was 77.65% and 78.59%, respectively. In total, 8 out of 52 cell lines showed sensitivity to this structure, which indicates an expansion of the spectrum of antitumor action due to the change in structure. Compound **5**, which introduced a piperidine fragment, demonstrated pronounced cytotoxicity against **SK-MEL-28** melanoma cells, where the inhibition level reached 84.37%. The most sensitive cell lines to the action of compound **8** were **OVCAR-4** (ovarian cancer), **786-0** and **TK-10** (kidney cancer), **SF-539** and **SNB-75** (central nervous system tumors), **EKVX** and **NCI-H522** (non-small cell lung cancer). In these cases, the growth inhibition ranged from –80.26% to –125.90%, indicating a pronounced cytotoxic effect of the compound.

Within the framework of the study, compound **8** was selected for in-depth biological testing on a panel of 60 human cancer cell lines (NCI-60) at five concentrations in the range of 10^{-4}–10^{-8} M, as it demonstrated the highest activity among all tested samples. The following key parameters were used to assess the biological activity of the compound: GI_{50} (Growth Inhibition 50%) – concentration that inhibits cell growth by 50% (cytostatic effect); TGI (Total Growth Inhibition) – concentration that completely stops cell proliferation (cytostatic effect); LC_{50} (Lethal Concentration 50%) – concentration that causes the death of 50% of cells (cytotoxic effect) (Table 2).

The results of the in-depth screening showed that compound **8** demonstrated a pronounced cytostatic effect against most cell lines. The lowest GI_{50} values (less than 1 μM) were recorded for the following lines: **OVCAR-4** (ovarian cancer): $GI_{50} = 0.382$ μM, **SR** (lymphoma): $GI_{50} = 0.676$ μM, **786-0** (renal cancer): $GI_{50} = 0.696$ μM, **A498** (renal cancer): $GI_{50} = 0.528$ μM, **BT-549** (breast cancer): $GI_{50} = 0.666$ μM. For these cell lines, a decrease in viability (LC_{50}) was also recorded within the range of 50 μM, which confirms the cytotoxic effect of compound (Table 2).

To quantify the selectivity of compound **8**, selectivity indices (SI) were calculated by dividing the mean values of GI_{50}, TGI and LC_{50} across the entire cell panel (MG_MID) by the corresponding values of each parameter for a specific cell line. The resulting ratios were interpreted according to the following scale: SI from 3 to 6 – moderate selectivity; SI > 6 – high selectivity; SI < 3 – no selectivity.

Table 1

In vitro antitumor activity of thiopyrano[2,3-d]thiazole derivatives (**3.1, 3.2**, **5**, **8**) at a fixed concentration of 10^{-5} M.

Comp.	Mean growth, %	Range of growth, %	Top 10 most sensitive cell lines/growth %	Positive cytostatic effect[a]	Positive cytotoxic effect[b]
3.1	90.78	40.55–127.56	CCRF-CEM (Leukemia) 75.33. K-562 (Leukemia) 68.93. MOLT-4 (Leukemia) 69.56. EKVX (Non-Small Cell Lung Cancer) 60.22. NCI-H226 (Non-Small Cell Lung Cancer) 75.50. SK-MEL-5 (Melanoma) 68.62. SN12C (Renal Cancer) 73.95. MCF7 (Breast Cancer) 74.79. HS 578T (Breast Cancer) 40.55. T-47D (Breast Cancer) 46.69	0/52	2/52
3.2	78.16	21.41–132.16	CCRF-CEM (Leukemia) 49.82. K-562 (Leukemia) 49.01. MOLT-4 (Leukemia) 45.81. SR (Leukemia) 37.91. EKVX (Non-Small Cell Lung Cancer) 44.68. NCI-H226 (Non-Small Cell Lung Cancer) 56.17. SK-MEL-5 (Melanoma) 49.19. MCF7 (Breast Cancer) 57.28. HS 578T (Breast Cancer) 22.35. T-47D (Breast Cancer) 21.41	0/52	8/52
5	50.25	–84.37–94.31	HL-60(TB) (Leukemia) 21.57. K-562 (Leukemia) 23.79. SR (Leukemia) 12.64. NCI-H460 (Non-Small Cell Lung Cancer) 23.47. HCT-15 (Colon Cancer) 28.05. HT29 (Colon Cancer) 16.45. SF-539 (CNS Cancer) 10.42. MALME-3M (Melanoma) 16.54. SK-MEL-28 (Melanoma) –84.37. UACC-257 (Melanoma) –1.83	2/57	22/57
8	52.60	–80.2–125.90	SR (Leukemia) –25.15. EKVX (Non-Small Cell Lung Cancer) –4.93. NCI-H522 (Non-Small Cell Lung Cancer) –1.78. KM12 (Colon Cancer) 10.35. SF-539 (CNS Cancer) –80.26. SNB-75 (CNS Cancer) –27.19. OVCAR-4 (Ovarian Cancer) –28.76. 786-0 (Renal Cancer) –0.99. TK-10 (Renal Cancer) –20.94. HS 578T (Breast Cancer) –1.72	9/52	25/52

Table 2

Summary of NCI-60 screening results for compound **8** at multiple concentrations (10^{-4}–10^{-8} M)

Cell Line	GI_{50}, μM / SI	TGI, μM / SI	LC_{50}, μM / SI
1	2	3	4
Leukemia			
CCRF-CEM	> 100/0.08	> 100/0.1	> 100/0.12
HL-60(TB)	> 100/0.08	> 100/0.1	> 100/0.12
K-562	> 100/0.08	> 100/0.1	> 100/0.12
MOLT-4	> 100/0.08	> 100/0.1	> 100/0.12
RPMI-8226	22.20/0.36	3.88/2.57	67.60/0.185
SR	0.676/11.83	20.80/0.48	53.50/0.23
MG_MID	73.81	70.78	86.85
Non-small cell lung cancer			
A549/ATCC	36.60/0.218	> 100/0.1	> 100/0.12
EKVX	36.50/0.219	> 100/0.1	> 100/0.12
HOP-62	> 100/0.08	> 100/0.1	> 100/0.12
HOP-92	11.40/0.7	25.20/0.39	55.60/0.22
NCI-H226	> 100/0.08	> 100/0.1	> 100/0.12

Continuation of Table 2

1	2	3	4
NCI-H23	> 100/0.08	> 100/0.1	> 100/0.12
NCI-H322M	> 100/0.08	> 100/0.1	> 100/0.12
NCI-H460	17.10/0.46	33.80/0.29	67.20/0.186
NCI-H522	43.20/0.18	> 100/0.1	> 100/0.12
MG_MID	60.53	84.33	91.42
Colon cancer			
COLO 205	> 100/0.08	> 100/0.1	> 100/0.12
HCC-2998	> 100/0.08	> 100/0.1	> 100/0.12
HCT-116	11.40/0.7	23.80/0.4	49.70/0.25
HCT-15	> 100/0.08	> 100/0.1	> 100/0.12
HT29	35.10/0.22	> 100/0.1	> 100/0.12
KM12	13.50/0.59	27.10/0.36	54.30/0.23
SW-620	42.70/0.18	> 100/0.1	> 100/0.12
MG_MID	57.52	78.7	86.2
CNS cancer			
SF-268	13.40/0.59	26.30/0.38	51.60/0.24
SF-295	> 100/0.08	> 100/0.1	> 100/0.12
SF-539	18.50/0.43	40.30/0.24	87.70/0.14
SNB-19	> 100/0.08	> 100/0.1	> 100/0.12
SNB-75	12.70/0.62	26.40/0.37	55.100.22
U251	11.90/0.67	27.80/0.35	65.30/0.19
MG_MID	42.75	53.46	76.6
Melanoma			
LOX IMVI	55.60/0.14	> 100/0.1	> 100/0.12
MALME-3M	> 100/0.08	> 100/0.1	> 100/0.12
M14	27.90/0.28	54/0.18	> 100/0.12
MDA-MB-435	19.10/0.41	35.90/0.27	67.30/0.18
SK-MEL-2	18.90/0.42	38/0.26	76.40/0.16
SK-MEL-28	> 100/0.08	> 100/0.1	> 100/0.12
SK-MEL-5	> 100/0.08	> 100/0.1	> 100/0.12
UACC-257	30.90/0.25	68.20/0.14	> 100/0.12
UACC-62	> 100/0.08	> 100/0.1	> 100/0.12
MG_MID	61.37	77.34	93.7
Ovarian cancer			
IGROV1	> 100/0.08	> 100/0.1	> 100/0.12
OVCAR-3	17.60/0.45	32.50/0.28	59.70/0.21
OVCAR-4	0,382/20.94	0,864/11.57	29.5/0.42
OVCAR-5	> 100/0.08	> 100/0.1	> 100/0.12
OVCAR-8	14.6/0.54	28/0.35	53.70/0.23
NCI/ADR-RES	99.30/0.08	> 100/0.1	> 100/0.12
SK-OV-3	> 100/0.08	> 100/0.1	> 100/0.12
MG_MID	61.69	65.9	77.55
Renal cancer			
786-0	0.696/11.49	38.80/0.25	> 100/0.12
A498	0,528/15.1	15.80/0.63	40.60/0.30
ACHN	–	–	–
CAKI-1	16.70/0.47	31.80/0.31	60.30/0.20
RXF 393	64.70/0.12	> 100/0.1	> 100/0.12
SN12C	18.30/0.43	42.60/0.23	99.60/0.12
TK-10	15.40/0.51	34.10/0.29	75.50/0.16
UO-31	> 100/0.08	> 100/0.1	> 100/0.12
MG_MID	30.90	51.87	82.28
Prostate cancer			
PC-3	18/0.44	33.20/0.3	61.40/0.20
DU-145	22.60/0.35	43.60/0.22	84.10/0.14
MG_MID	29.30	38.4	72.75
Breast cancer			
MCF7	> 100/0.08	> 100/0.1	> 100/0.12

Continuation of Table 2

1	2	3	4
MDA-MB-231/ATCC	29.50/0.27	98.20/0.101	> 100/0.12
HS 578T	14.70/0.54	28.10/0.35	53.40/0.23
BT-549	0,666/12	17.80/0.56	42.90/0.29
T-47D	> 100/0.08	> 100/0.1	> 100/0.12
MDA-MB-468	> 100/0.08	> 100/0.1	> 100/0.12
MG_MID	57.47	74	72.71
MG_MID_60	8	10	12.54

According to the results presented in Table 2, most of the SI values were less than 3, indicating limited selectivity for most cell lines. At the same time, the highest SI values were recorded for the cells that showed the highest sensitivity: OVCAR-4 (ovarian cancer): SI = 20.94 (GI_{50}), SI = 11.57 (TGI); SR (leukemia): SI = 11.83 (GI_{50}); A498 (kidney cancer): SI = 15.1 (GI_{50}); 786-0 (kidney cancer): SI = 11.49 (GI_{50}); BT-549 (breast cancer): SI = 12.0 (GI_{50}) (Table 2, Fig. 4).

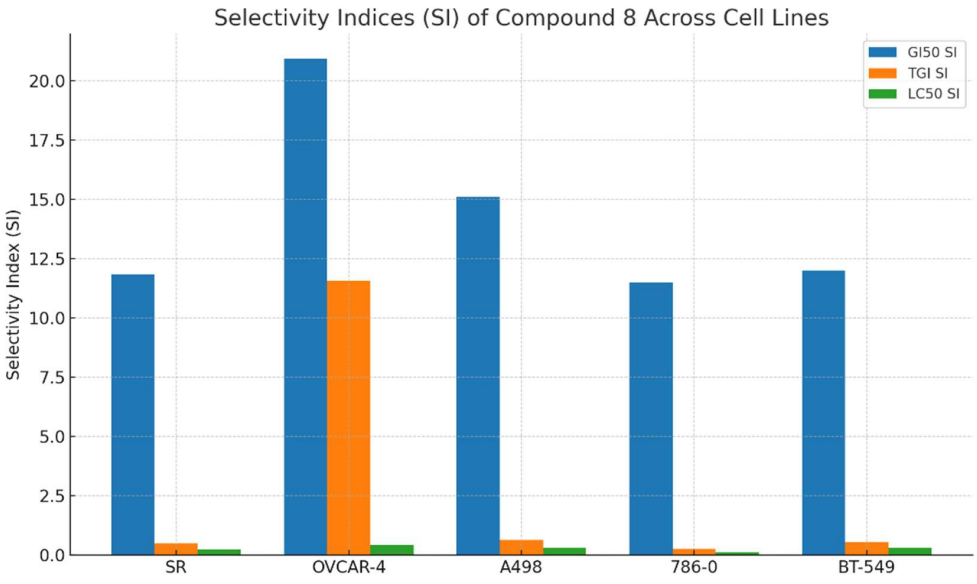

Fig. 4. Comparison of SI by GI_{50}, TGI and LC_{50} parameters for cell lines with the highest sensitivity to compound **8**

5. Discussion

A series of thiopyrano[2,3-d]thiazole derivatives was synthesized via a two-step domino sequence involving Knoevenagel condensation followed by intramolecular heterocyclization through a hetero-Diels-Alder mechanism between 4-thioxo-2-oxothiazolidinone and *O*-alkylated salicylaldehyde derivatives bearing allylic or propargylic substituents. This cascade approach is highly valuable in medicinal chemistry, as it enables the rapid and efficient construction of fused heterocyclic frameworks that serve as privileged scaffolds for the development of novel bioactive molecules. Such strategies minimize the number of synthetic steps and improve the overall atom economy of the process. Subsequent *N*3-alkylation furnished compounds **3.1** (60.0%), **3.2** (67.0%), and **4** (58.0%). The moderate yields observed may be attributed to the partial reversibility of the alkylation step, the formation of side products, or the instability of reaction intermediates under basic ethanol conditions (KOH). Nevertheless, the selected conditions were optimized to preserve the integrity of the thiopyrano[2,3-d]thiazole core. The introduction of a piperidine moiety enabled the synthesis of a water-soluble methanesulfonate salt **5** (70.0%), which significantly enhances the pharmacokinetic potential of the molecule. Improved aqueous solubility is a critical parameter for the bioavailability of prospective drug candidates and expands their applicability in biological assays. Notably, the reaction with 2,5-(2-propynyloxy)benzaldehyde led to *in situ* aromatization and afforded a conformationally rigid, planar molecule-compound **8**. The resulting extended π-delocalization and molecular planarity are favorable for interactions with biological targets through π-π stacking, hydrogen bonding, and reduced entropic penalty upon binding to enzyme active sites or receptor domains. This structural organization may play a key role in achieving high binding affinity and selectivity, as confirmed by the results of the NCI-60 screening.

Biological testing of the synthesized thiopyrano[2,3-d]thiazole derivatives revealed certain structure–activity relationships and emphasized the impact of minor structural modifications on the cytotoxicity profile of the compounds. Specifically, compound **3.1** exhibited moderate cytostatic activity only against two breast cancer cell lines (**HS 578T** and **T-47D**), indicating a narrow spectrum of activity and suggesting a possible specificity in its mechanism of interaction. A positional change of the chlorine atom from the ortho- (**3.1**) to the meta-position (**3.2**) of the aromatic ring led to increased inhibitory activity and a broader spectrum of cytotoxicity for **3.2**. This highlights the critical role of electronic and steric effects in shaping the biological activity of these molecules. The introduction of a piperidine fragment (compound **5**) enhanced cytotoxicity, particularly against the **SK-MEL-28** melanoma cell line (84.37% growth inhibition). In total, 22 out of 57 tested cell lines were sensitive

to this compound, demonstrating a broad activity profile with partial selectivity toward melanoma cells. This suggests that cyclic amines may improve membrane permeability and target engagement through hydrophobic and hydrogen bonding interactions. Compound **8** exhibited the broadest anticancer activity, inducing cancer cell death in 25 out of 52 tested cell lines. The most sensitive lines included **OVCAR-4** (ovarian cancer), **786-0** and **TK-10** (renal cancer), **SF-539** and **SNB-75** (central nervous system tumors), **EKVX**, and **NCI-H522** (non-small cell lung cancer). The overall results of the primary screening confirm the high potential of compound **8** as a promising antitumor agent, due to its broad cytotoxic spectrum and pronounced effect across cancer cells of diverse histogenesis.

Further in-depth evaluation using the NCI-60 panel confirmed that compound **8** exhibited high activity in the micromolar range. The lowest GI_{50} values (<1 μM) were recorded for **OVCAR-4** (0.382 μM), **SR** (lymphoma, 0.676 μM), **786-0** (0.696 μM), **A498** (0.528 μM), and **BT-549** (0.666 μM), meeting the criteria for potential antitumor agents. Corresponding LC_{50} values also confirmed the compound's ability to induce cancer cell death. Compound **8** demonstrated the highest selectivity indices (SI) for **OVCAR-4** (SI = 20.94), **A498** (15.1), **786-0** (11.49), **SR** (11.83), and **BT-549** (12.0), suggesting its potential targeted action against ovarian, renal, hematopoietic, and aggressive breast cancer cell lines.

In order to provide a generalized assessment of the biological activity of compound **8** on the entire NCI-60 cell panel, the average values of three key biological indicators were analyzed: GI_{50} (Growth Inhibition 50%) - the average value was 8 μM, which indicates a pronounced inhibition of proliferation of most cancer cells at moderate concentrations of the test compound; TGI (Total Growth Inhibition) - the average value of 10 μM demonstrates the ability of the compound to completely stop cell growth (cytostatic effect) in most cases; LC_{50} (Lethal Concentration 50%) - the average value of 12.54 μM confirms the cytotoxic effect on a significant part of the cell lines. These values were obtained based on MG_MID (Mean Graph Midpoint), which is a standardized indicator of the average efficacy of the compound across all 60 cell lines. This approach allows for an objective comparison of activity between different molecules or structures, regardless of the tumor type.

Practical relevance. The practical relevance of the study lies in the development of an efficient synthetic strategy for constructing novel thiopyrano[2,3-*d*]thiazole derivatives with pronounced antitumor potential. Compound **8** exhibited high cytotoxicity and selectivity, making it a promising candidate for further evaluation.

Research limitations. The main limitation of the study is the restricted number of synthesized derivatives, as well as the lack of *in vivo* studies and mechanistic investigations of their molecular targets.

Prospects for further research. Future research should focus on expanding the series of analogues, elucidating their mechanisms of action, applying computational modelling of target interactions, and evaluating pharmacokinetics and toxicity *in vivo.*

6. Conclusions

As a result of the study, five new thiopyrano[2,3-*d*]thiazole derivatives **3.1**, **3.2**, **4**, **5** and **8** were synthesized by Knoevenagel domino condensation-hetero-Diels-Alder-cyclization with subsequent N3-alkylation, which allowed obtaining structurally diverse products with high chemical efficiency. The introduction of a piperidine fragment made it possible to synthesize a water-soluble methanesulfonate salt **5**. Primary biological screening demonstrated the promise of several synthesized compounds as antitumor agents, among which compound **8** was the most active, exhibiting pronounced cytotoxicity and selectivity against ovarian (**OVCAR-4**), kidney (**786-0**, **A498**), lymphoma (**SR**) and breast (**BT-549**) cancer cells. The results of the full NCI-60 screening confirmed that compound **8** has a broad spectrum of activity and can inhibit the growth and causing death of cancer cells at micromolar concentrations, which allows us to consider it as a promising candidate for further preclinical study in the context of the development of new anticancer agents.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this research, whether financial, personal, or authorship-related, that could affect the research and its results presented in this article.

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Data availability

The manuscript has no associated data.

Use of artificial intelligence

The authors did not use any artificial intelligence technologies in the preparation of this study.

Authors' contributions

Mykhailo Hoidyk: Investigation, Methodology, Data Curation; **Andriy Karkhut**: Formal Analysis, Validation, Resources; **Svyatoslav Polovkovych**: Conceptualization, Writing-Original Draft, Project Administration; **Roman Lesyk**: Supervision, Writing-Review & Editing, Conceptualization.

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Mykhailo Hoidyk*, PhD Student, Department of Pharmaceutical, Organic and Bioorganic Chemistry, Danylo Halytsky Lviv National Medical University, Pekarska str., 69, Lviv, Ukraine, 79010

Andriy Karkhut, PhD, Associate Professor, Department of Technology of Biologically Active Substances, Pharmacy and Biotechnology, Lviv Polytechnic National University, Stepana Bandery str., 12, Lviv, Ukraine, 79013

Sviatoslav Polovkovych, Doctor of Chemical Sciences, Professor, Department of Technology of Biologically Active Substances, Pharmacy and Biotechnology, Lviv Polytechnic National University, Stepana Bandery str., 12, Lviv, Ukraine, 79013

Roman Lesyk, Doctor of Pharmaceutical Sciences, Professor, Department of Pharmaceutical, Organic and Bioorganic Chemistry, Danylo Halytsky Lviv National Medical University, Pekarska str., 69, Lviv, Ukraine, 79010

Corresponding author: *Mykhailo Hoidyk, e-mail: mgojdik@gmail.com*