scientific reports

Check for updates

OPEN

Exploration of 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylic acid derivatives effect on triple-negative breast, prostate cancer and melanoma cell 2D and 3D cultures

Guoda Pranaitytė[1], Birutė Grybaitė[1], Ugne Endriulaityte[2], Vytautas Mickevičius[1]✉ & Vilma Petrikaitė[2,3]✉

1-Substituted 5-oxopyrrolidine-3-carboxylic acid and its derivatives play an important role as components of many biologically active molecules. This study describes the synthesis of 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylic acid derivatives and their anticancer properties. The target compounds were prepared using 2,4-difluoroniline as a starting material; in this way, derivatives of benzimidazoles, hydrazones and azoles were formed. Investigation of the anticancer activity of all synthesized compounds showed that the hydrazones had the strongest effect on cancer cell lines. Compounds were tested for their cytotoxic effect by the MTT assay in human triple-negative breast cancer MDA-MB-231, prostate adenocarcinoma PPC1, melanoma A375 and human foreskin fibroblasts CRL-4001 after 72 hours of incubation. The impact of the compounds on cancer cell migration was assessed using a 'wound healing assay'. Activity in 3D cultures was determined by evaluating changes in spheroid size and assessing cell viability. Overall, the selected compounds 7b, 9c, 9e, 9f and 10 exhibited greater activity in the A375 cell line and were less active against the MDA-MB-231 cell line. Compounds 9c, 9e and 10 showed relatively higher selectivity for cancer cells over fibroblasts. Hydrazone 9f, bearing *N*′-(4-methylbenzylidene) moiety, was identifiedasthe most cytotoxic compound in both prostate adenocarcinoma PPC-1 and melanoma A375 cells in monolayer and 3D culture models. Compound 9e, with *N*′-(4-bromobenzylidene) moiety, exhibited the most pronounced inhibitory effect on cell migration as determined by the 'wound healing' assay.

Keywords Azoles, Hydrazones, Triple-negative breast cancer, Prostate carcinoma, Melanoma, Cytotoxicity, Cell migration, Tumor spheroids

Cancer remains one of the major public health challenges today. According to data from the World Health Organization, nearly 10 million people died from this disease in 2020[1]. Chemotherapy is the most common form of cancer treatment; however, it also affects healthy cells and is associated with serious side effects. Treatment with low molecular weight compounds that target specific enzymes or receptors is becoming increasingly popular, as these agents block oncogenic signalling pathways and interfere with cancer cell proliferation[2]. Consequently, the search for novel synthetic molecules with high activity and low toxicity has become an important key focus in the development of new therapies for malignant tumors.

β-Amino acid derivatives are of considerable interest in the field of organic synthesis due to their broad spectrum of biological activity. These compounds play a role in metabolic and immune system regulatory processes, and are therefore used in the treatment of diabetes, cardiovascular diseases and other ailments[2,3].

[1]Kaunas University of Technology, Radvilėnų Rd. 19, Kaunas LT-50254, Lithuania. [2]Laboratory of Drug Targets Histopathology, Institute of Cardiology, Lithuanian University of Health Sciences, Sukileliu pr. 13, Kaunas LT-50162, Lithuania. [3]Institute of Biotechnology, Life Sciences Center, Vilnius University, Saulėtekio Ave. 7, Vilnius LT-10257, Lithuania. ✉email: vytautas.mickevicius@ktu.lt; vilma.petrikaite@lsmuni.lt

For example, five-membered 2-pyrrolidinones represent a class of heterocyclic compounds with a wide variety of biologically active derivatives. This structural motif is common in many natural products that exhibit diverse biological effects. 2-Pyrrolidinone-based compound pyrrocidine A is a known antimicrobial agent produced by the endophytic fungus *Sarocladium zeae*[4]. Its significant role is highlighted not only by its antimicrobial properties[5–7] but also by its potent cytotoxic activity against human acute promyelocytic leukemia HL60 cells. This activity is approximately 70-fold higher than that of its analog, pyrrocidine B, which lacksthe α,β-unsaturated carbonyl group[4].

Hydrazones, due to their significant biological potential, are increasingly attracting the attention of researchers involved in the discovery and development of effective pharmaceuticals. This fragment is utilized in the synthesis of compounds with anticonvulsant, analgesic, anticancer, and antiviral properties[8–10]. Compounds containing a hydrazone moiety have demonstrated activity against gastric, lung and breast cancer cell lines without exerting toxic effects on normal liver cell lines. Therefore, these compounds represent promising candidates for the development of novel anticancer agents. Additionally, the hydrazone group enables the formation of hydrogen bonds, which enhances interactions between organic molecules and amino acids – the primary components of biological targets[9,11].

Our inclusion of the 2,4-difluorophenyl fragment in the molecule was guided by the understanding that fluorine atoms play a distinct and significant role in influencing molecular conformation. From a steric perspective, the impact of fluorine is expected to be minimal, as it is a small atom with a van der Waals radius of 1.47 Å—comparable to that of hydrogen (1.20 Å). Additionally, fluorine's high electronegativity results in strongly polarized C–F bonds, which can create opportunities for hydrogen bonding interactions with biological targets. Furthermore, several FDA-approved fluorinated drugs—such as nirogacestat (approved for the treatment of progressing desmoid tumors), alpelisib (for breast cancer), and sotorasib (for non-small cell lung cancer)—demonstrate the clinical relevance and therapeutic potential of fluorine-containing compounds in modern drug development. For these reasons, we have incorporated the 2,4-difluorophenyl fragment into our molecules[12,13].

To evaluate the anticancer activity of the synthesized 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylic acid derivatives, several cancer types were selected. Triple-negative breast cancer (TNBC) is a highly aggressive and metastatic subtype, characterized by a high recurrence rate and mortality[14]. The MDA-MB-231 is commonly employed as a model system for screening compounds targeting this cancer type. The limited therapeutic options for TNBC highlight the urgent need for more effective and less toxic novel agents.

Although prostate carcinoma is generally considered less lethal, it remains the second most frequently diagnosed cancer in men, and certain metastatic subtypes exhibit resistance to current treatment strategies[15]. The PPC-1 cell line was selected for this study due to its well-established metastatic properties and widespread use as a model for evaluating compound efficacy against prostate carcinoma[16].

Melanoma is the most lethal form of skin cancer, and its associated mortality continues to rise each year[17]. Although therapeutic advances have accelerated since 2011, the high aggressiveness of melanoma and the frequent development of treatment resistance underscore the urgent need for novel therapeutic options[18]. Depending on the melanoma subtype, disease stage, patient health status, and other factors, a range of treatment strategies may be employed, including surgery, radiotherapy, chemotherapy, and drug combinations – such as immune checkpoint inhibitors and targeted therapies[19]. However, the primary chemotherapeutic agent used to treat metastatic melanoma remains the non-specific alkylating agent dacarbazine[20]. The A375 cell line, known for its aggressive and rapidly proliferating phenotype[21], was therefore included in this study to identify potential candidate compounds for melanoma traetment.

For screening the synthesized compounds, we chose to assess their cytotoxicity using the MTT assay, a widely employed method in the initial stages of in vitro activity evaluation due to its simplicity[22]. Additionally, considering the metastatic potential of the selected cancer types, the effects of the compounds on cell migration were evaluated using the 'wound healing' assay. Finally, given that three-dimensional (3D) cell cultures more accurately represent the tumor microenvironment compared to traditional monolayer cultures, this model was employed chosen for a more detailed analysis of the most active candidate compounds.

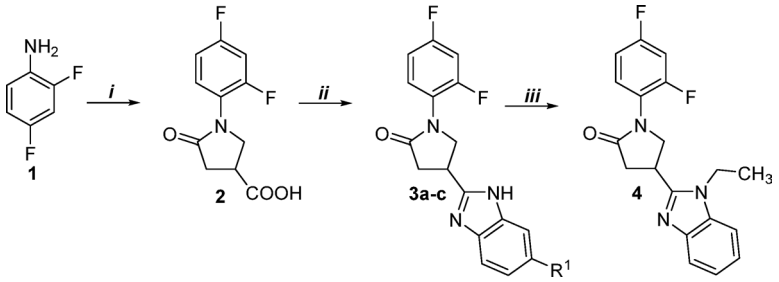
Results and discussion

Chemistry

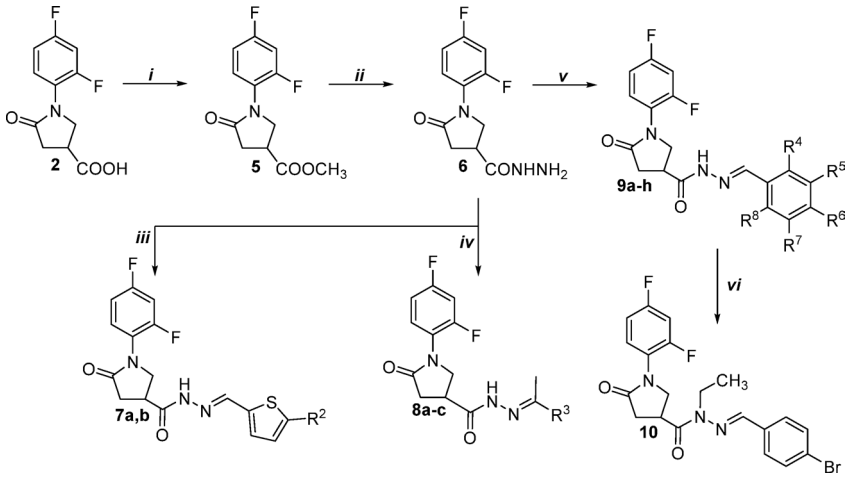
In our previous studies, we reported the synthesis and in vitro anticancer activity of 5-oxo-1-arylpyrrolidine-3-carbohydrazide derivatives[23]. The results were promising for further exploration of this scaffold to enhance the anticancer activity and establish the structure–activity relationship (SAR) properties of 5-oxo-1-arylpyrrolidine-3-carbohydrazide derivatives. In the present work, 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylic acid (**2**) was prepared according to the established method[22], by refluxing 2,4-difluoroaniline (**1**) with itaconic acid in water. The intermediate product – 4-arylamino-3-carboxybutanoic acid – was not isolated from the reaction mixture because it lost a water molecule and underwent cyclization to form 3-carboxy-5-oxopyrrolidine (**2**) during the reaction (Scheme 1).

The target compounds **3a-c** were synthesized by the Philips method (heating of both reagents in 4 M hydrochloric acid), and a sufficient yield of benzimidazoles was obtained. *N*-Substituted benzimidazole **4** was synthesized by alkylation of 4-(1*H*-benzo[*d*]imidazol-2-yl)-1-(2,4-difluorophenyl)pyrrolidine-2-one **3a** with iodoethane in DMF in the presence of potassium carbonate and potassium hydroxide. In the ^{1}H NMR spectrum of the alkylated compound **4**, compared to the spectrum of the non-alkylated compound **3a**, no proton signal characteristic of the NH fragment of the imidazole ring is observed in weak magnetic fields, and proton signals of the ethyl group appear at 1.33 and 2.88–3.06 ppm. In the ^{13}C NMR spectrum of the alkylated compound **4**, the signals of the carbon atoms of the ethyl group are observed at 15.20 and 29.76 ppm.

Methyl 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylate (**5**) was synthesized by esterification of acid **2** with an excess of methanol under reflux in the presence of a catalytic amount of sulphuric acid. The reaction



Scheme 1. Synthesis of compounds **2–4**. **3a** R^1 = H; **3b** R^1 = F; **3c** R^1 = Cl. Reagents and conditions: (i) itaconic acid, water, Δ, 22 h, 73%; (ii) corresponding *o*-phenylenediamine, 4 M HCl, Δ, 72 h, 5% Na_2CO_3, H_2O, 36–49%; (iii) CH_3CH_2I, KOH, K_2CO_3, DMF, RT, 1 h, 68%.



Scheme 2. Synthesis of compounds **5–10**. **7a** R^2 = H; **7b** R^2 = NO_2; **8a** R^3 = CH_3; **8b** R^3 = C_2H_5; **8c** R^3 = 4-NH_2-C_6H_4; **9a** R^4, R^5, R^6, R^7, R^8 = H; **9b** R^4, R^5, R^7, R^8 = H, R^6 = F; **9c** R^4, R^5, R^7, R^8 = H, R^6 = Cl; **9d** R^4, R^5, R^7 = H, R^6, R^8 = F; **9e** R^4, R^5, R^7, R^8 = H, R^6 = Br; **9f** R^4, R^5, R^7, R^8 = H, R^6 = CH_3; **9 g** R^4, R^5, R^7, R^8 = H, R^6 = $N(CH_3)_2$; **9 h** R^4, R^5, R^7, R^8 = H, R^6 = NO_2. Reagents and conditions: (i) MeOH, H_2SO_4, Δ, 2 h, 76%; (ii) $N_2H_4.H_2O$, Δ, 8 h, *i*-PrOH, 62%; (iii) coresponding thiophene-2-carbaldehyde, *i*-PrOH, Δ, 2 h, 72% (**7a**), 96% (**7b**); (iv) acetone or ethyl methyl ketone, CH_3COOH, Δ, 1 h, 85% (**8a**), 65% (**8b**) or 4'-aminoacetophenone, CH_3COOH, *i*-PrOH, Δ, 5 h, 56% (**8c**); (v) coresponding aromatic aldehyde, *i*-PrOH, Δ, 1 h, 70–92%; (vi) CH_3CH_2I, KOH, K_2CO_3, DMF, RT, 1 h, 54%.

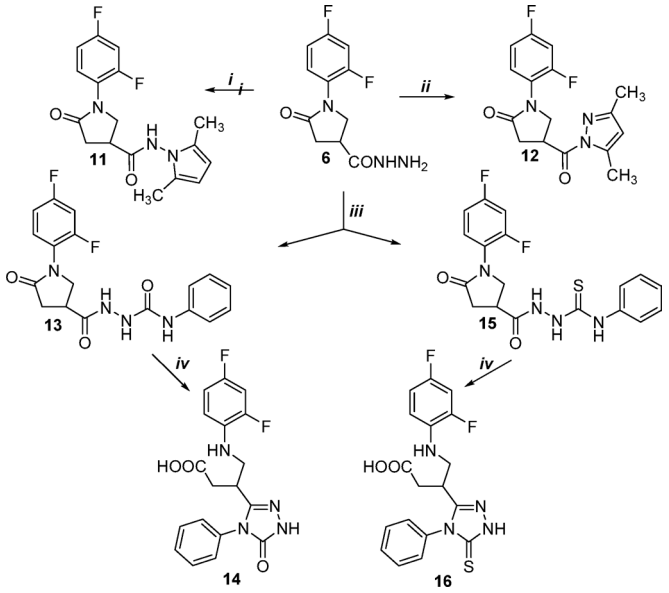
of ester **5** with hydrazine hydrate in 2-propanol under reflux yielded 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**6**), which crystallized from the reaction mixture upon cooling (Scheme 2). Condensation of hydrazide **6** with heterocyclic, aromatic aldehydes and ketones produced hydrazones **7–9**. The hydrazones gave rise to two sets of spectral lines (intensity ratio ~ 35:65) in the ^{1}H and ^{13}C NMR spectra (DMSO-d_6). Thus, the hydrazones form stable *E/Z* isomers due to the restricted rotation of the amide group around the CO-NH bond. This has been discussed in more detail in previous works[23,24].

Alkylation of *N*'-(4-bromobenzylidene)-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**9e**) with iodoethane was investigated. The reaction was carried out in a large excess of iodoethane in the presence of potassium hydroxide and potassium carbonate, and compound **10** was synthesized. The absorption band characteristic of the NH group is absent in the IR spectra compared to the initial compounds.

The reaction of acid hydrazides with *β*- and *γ*-diketones usually provides cyclic compounds (Scheme 3). The condensation of hydrazide **6** with 2,5-hexanedione in 2-propanol in the presence of a catalytic amount of acetic acid resulted in the formation of 1-(2,4-difluorophenyl)-*N*-(2,5-dimethyl-1*H*-pyrrol-1-yl)-5-oxopyrrolidine-3-carboxamide (**11**). The intense singlets at 1.91–2.11 and 5.67 ppm, attributed to the CH_3 and CH groups of the pyrrole ring, were present in the ^{1}H NMR spectra. The double intensity resonances at 10.88, 103.10, and 126.73 ppm in the ^{13}C NMR spectra pointed to the existence of a pyrrole ring.

The condensation of hydrazide **6** with 2,4-pentanedione in 2-propanol in the presence of a catalytic amount of hydrochloric acid resulted in the formation of pyrazole **12**. The ^{13}C NMR spectra of this compound exhibited three resonances at 111.15-111.48, 143.45, and 151.73 ppm, assigned to the pyrazole ring.

The interaction of carbohydrazide **6** with phenyl isocyanate and phenyl isothiocyanate in methanol under reflux gave (thio)semicarbazides **13**, **15**. A precipitate formed during the reaction. In the ^{1}H NMR spectrum of compound **15**, three singlets at 9.58, 9.85 and 10.18 ppm, show the presence of three NH groups. The formation



Scheme 3. Synthesis of compounds **11–16**. Reagents and conditions: (i) Hexane-2,5-dione, *i*-PrOH, CH_3COOH, Δ, 2 h, 68%; (ii) Pentane-2,4-dione, *i*-PrOH, HCl, Δ, 2 h, 39%; (iii) PhNCO (**13**) or PhNCS (**15**), MeOH, Δ, 3 h, 81% (**13**), 61% (**15**); (iv) 4% NaOH, Δ, dil. HCl pH 6, 19 h (**14**) or CH_3COOH pH 6, 2 h (**16**) 43% (**14**), 71% (**16**).

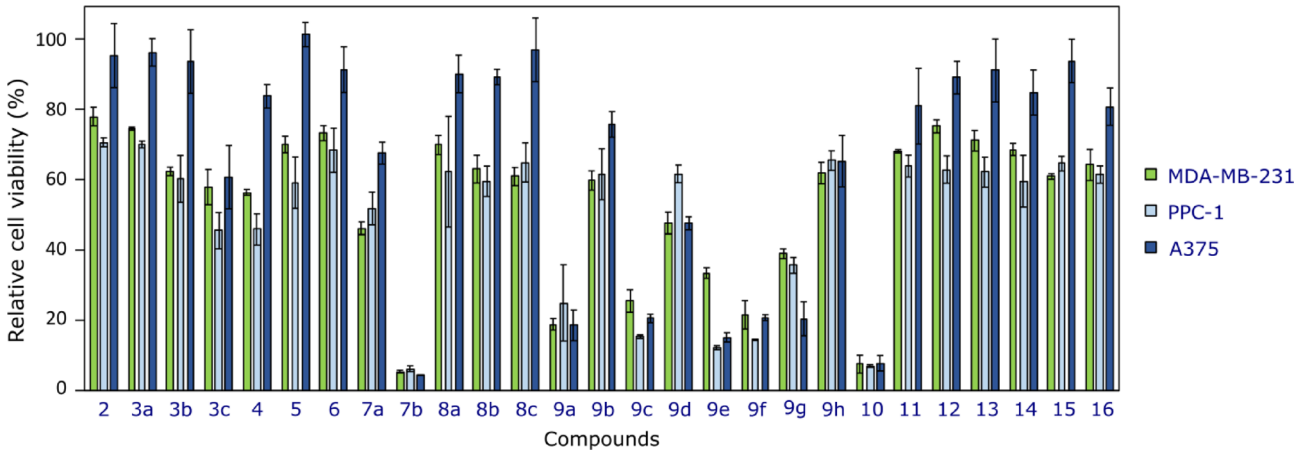


Fig. 1. Effect of compounds **2–16** on cancer cell viability at 100 μM concentration against human triple-negative breast cancer MDA-MB-231, human prostate carcinoma PPC-1, and human melanoma A375 cell lines, $n = 3$. The effect was established after 72 h of incubation by MTT assay.

of the C(O)-NH-NH-C(S)-NHPh fragment is further confirmed by the resonances at 172.04 and 181.16 ppm assigned to C = O and C = S groups and additional spectral lines in the aromatic region in the ^{13}C NMR spectrum.

The final step of the study involved ring closure reactions, in which (thio)semicarbazides **13**, **15** were heated under reflux in aqueous sodium hydroxide solution. This reaction afforded 3-substituted 1,2,4-triazole derivatives; however, simultaneous opening of the pyrrolidinone cycle occured, forming the corresponding gamma amino acids **14**, **16**. Ring opening was confirmed by ^1H NMR spectra, where proton signals at 5.58 and 5.60 ppm were assigned to the NH fragment of the amino acids.

Biological activity

Cytotoxicity

The effect of compounds **2–16** on the viability of selected cancer cell lines varied considerably between both cell lines and compounds (Fig. 1). In general, the compounds were less active against the melanoma cell line, and only seven of them reduced the viability of A375 cells by more than 50%. The cytotoxicity against prostate and TNBC was comparable across different compounds. However, the same six compounds that showed the most

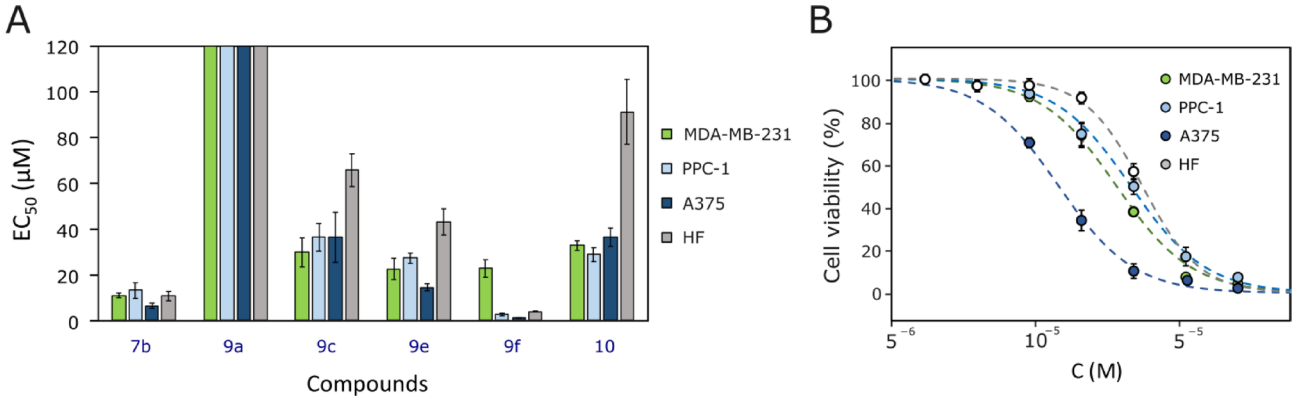


Fig. 2. EC_{50} values of the most active compounds **7b**, **9a**, **9c**, **9e**, **9f** and **10** after 72 h of incubation in human triple-negative breast cancer MDA-MB-231, human prostate carcinoma PPC, human melanoma A375 cell lines and human fibroblasts HF, established by the MTT assay, $n = 3$ (A). The dose-effect correlation has been calculated using the Hill equation (B). Data points are experimental values (averages of three repeats), while the lines are fit of the standard inhibition model with the Hill coefficient of 2.0 (MDA-MB-231, A375 and PPC-1 cells) and 2.6 (for HF).

Compound	EC_{50} (μM)			
	A375	MDA-MB-231	PPC-1	HF
7b	6.4 ± 1.1	10.9 ± 1.0	13.1 ± 3.4	10.6 ± 2.1
9a	275.9 ± 45.1	255.5 ± 49.3	455.2 ± 30.0	> 500.0
9c	36.6 ± 11.0	29.9 ± 6.4	36.6 ± 6.1	66.0 ± 7.2
9e	14.5 ± 1.5	22.6 ± 4.7	27.3 ± 2.3	43.3 ± 5.8
9f	1.0 ± 0.2	22.8 ± 3.9	2.5 ± 0.6	3.9 ± 0.2
10	36.6 ± 4.0	32.9 ± 2.1	28.9 ± 3.1	91.7 ± 14.3
Sunitinib	2.9 ± 0.99	3.4 ± 0.1	1.45 ± 0.07	8.17 ± 1.5
Doxorubicin	0.007 ± 0.0026	0.022 ± 0.0023	0.088 ± 0.01	0.33 ± 0.04
Cisplatin	8.13 ± 3.18	ND	27.8 ± 11.09	26.0 ± 10.8

Table 1. Established EC_{50} values for the most active compounds and anticancer drugs. ND – not determined.

substantial viability-reducing effect against melanoma cells – **7b**, **9a**, **9c**, **9e**, **9f**, and **10** - were also more active against the other two cell lines (The most active compound **7b** reduced A375 cell viability to as low as 4.41%.

The most active compounds were hydrazone derivatives bearing the *N*'-((5-nitrothiophen-2-yl) methylene) **(7b)**, *N*'-benzylidene **(9a)**, *N*'-(4-chlorobenzylidene) **(9c)**, *N*'-(4-bromobenzylidene) **(9e)**, *N*'-(4-methylbenzylidene) **(9f)** fragments, and alkylated hydrazone with *N*'-(4-bromobenzylidene) **(10)** moiety. Moreover, functionalization of the carboxyl group to heterocyclic compounds **2 – 6** and **11 – 16** resulted in a reduced anticancer activity.

The six most active compounds were included in further research, and their EC_{50} values against cancer cell lines and human fibroblasts were determined (Fig. 2). Compound **9a** was identified as non-toxic to fibroblasts and showed relatively low activity against all tested cancer cell lines, as its EC_{50} was higher than 200 μM.

Hydrazones **7b** and **9f** were identified as the most active compounds, particularly against the melanoma A375 and prostate carcinoma PPC-1 cell lines (EC_{50} values ranged from 1.0 to 13.1 μM). The activity of **7b** was similar across all tested cell lines, including cytotoxicity against fibroblasts. In contrast, compound **9f** exhibited significantly lower activity against the MDA-MB-231 cell line (it was from 11 to 22-fold less active compared to its activity against the other cancer cell lines). Notably, it was 3.9 times more selective towards A375 and 1.6 times more selective toward PPC-1 than fibroblasts. Compounds **9c**, **9e** and **10** were considerably less active against the tested cancer cells (EC_{50} values ranged rom 14.5 to 36.6 μM), but they demonstrated 1.6- to 3.0-fold selectivity for cancer cells over fibroblasts. Compound **9f**, bearing *N*'-(4-methylbenzylidene) moiety, was the most active against the melanoma cell line ($EC_{50} = 1.0 \pm 0.2$ μM) and the prostate adenocarcinoma cell line ($EC_{50} = 2.5 \pm 0.6$ μM). The most active compound against the TNBC cell line was hydrazone **7b**, bearing an *N*'-(5-nitrotiophen-2-yl) moiety ($EC_{50} = 6.4 \pm 1.1$ μM).

Additionally, we compared the cytotoxicity of the compounds with the kinase inhibitor sunitinib, the platinum derivative cisplatin and anthracycline-class drug doxorubicin (Table 1). These drugs have different mechanisms of action and are characterized by varying levels of cytotoxicity across different cancer cell lines. Sunitinib has been extensively studied in clinical trials for its potential in treating melanoma, metastatic breast cancer, prostate cancer and other cancer types[24]. Despite its high toxicity, cisplatin is frequently used as a first—

line chemotherapy, either alone or in combination with other anticancer drugs, to treat testicular cancer, head and neck cancer, lung cancer, breast cancer, melanoma, prostate cancer, and other malignancies[25]. Doxorubicin has been used to treat a wide range of cancers for more than five decades and remains a component of many chemotherapy regimens[26]. All the tested compounds were several hundred times less active than doxorubicin and several-fold less active than sunitinib, except compound **9f**, which exhibited approximately three-fold higher cytotoxicity against the A375 cell line than sunitinib (Table 1). Compound **9f** was also about ten-fold more active against the PPC-1 cell line compared to cisplatin. Additionally, the activity of compound **7b** was comparable to that of cisplatin against melanoma cells and was approximately two-fold more active against the prostate cancer cell line.

Overall, compounds **7b**, **9e** and **9f** demonstrated greater activity against the melanoma cell line than dacarbazine, a chemotherapeutic agent used in clinical practice to treat melanoma, whose EC_{50} values range from 25 to 100 μM in vitro[27]. However, newer compounds with significantly higher potency have been reported, such as the investigational proteasome inhibitor salinosporamide A (EC_{50} = 16.79 nM after 48 h)[28] and FDA-approved BRAF inhibitor dabrafenib (EC_{50} = 9.5 nM in colony formation assay)[29], approved in 2013 for the treatment of metastatic melanoma.

Our compounds were not more active than docetaxel, a drug approved for metastatic castration-resistant and hormone-sensitive prostate cancer, which exhibits nanomolar potency in PC-3 prostate cancer cells (EC_{50} = 15 ± 4 nM after 72 h of incubation).

Moreover, none of the tested compounds exhibited high activity against MDA-MB-231, which could be attributed to the intrinsic resistance and heterogeneity of this TNBC cell line[30]. The anthracycline drug doxorubicin, clinically used for the treatment of TNBC, has shown a wide range of activity in vitro, from nanomolar to micromolar concentrations[31,32], depending on variable experimental conditions. Considering the urgent need for more selective therapeutic options for TNBC, it may be hypothesized that the most active compound, hydrazone **7b**, holds potential for further investigation.

It should be emphasized that the cytotoxic activity determined in monolayer cultures represent only an initial step in compound development, as this in vitro model does not adequately reflect the complexity of the in vivo tumor microenvironment. Therefore, based on the observed selectivity and activity in the two-dimensional system, we proceeded to investigate the effects of these compounds on cancer cell migration, as well as their activity in a more physiologically relevant three-dimensional cell model (cancer cell spheroids).

Effect on cell migration

The most active and relatively more cancer cell-selective hydrazones – **7b**, **9c**, **9e** and **10** – were tested in a 'wound healing' assay at a concentration of 10 μM. To avoid confounding effects due to cytotoxicity, cell migration was evaluated up to 36 h for MDA-MB-231 and 48 h for PPC-1 and A375 cells (Fig. 3).

In general, none of the tested compounds inhibited TNBC cell (MDA-MB-231) migration after 12 and 24 h (Fig. 3, A and **D**). Only compound **9e**, after 36 h, exhibited a statistically significant anti-migratory effect compared to the control ($p < 0.05$). In contrast, compounds **7b**, **9c** and **9e** significantly inhibited prostate adenocarcinoma PPC-1 cell migration after 12 h of incubation (Fig. 3, B and **E**). These compounds also demonstrated inhibitory effect on melanoma A375 cell migration after 48 h; however, only compounds **9c** and **9e** showed a statistically significant effect ($p < 0.05$) (Fig. 3, C and **F**).

It should be noted that compound **10** did not exhibit any effect on the migration of the tested cell lines. Although compound **7b** demonstrated the highest cytotoxicity against all three cancer cell lines, it did not show superior activity in a 'wound-healing' assay compared to the other tested compounds. Interestingly, it was more active in PPC-1 cells even after a shorter incubation period, suggesting that its impact on cell migration is not directly linked to its cytotoxicity. Overall, compound **9e** emerged as the most active inhibitor of cell migration, exhibiting a variable but consistent anti-migratory effect across all cell lines at both earlier and later time points.

Overall, the effects of compounds on TNBC, prostate carcinoma and melanoma cell migration were inconsistent. It is important to acknowledge the limitations of the method used to assess cell migration. Although the 'wound healing' assay is widely recognised as a simple technique,, it is also associated with low reproducibility and technical challenges related to the creation of the scratch in the cell monolayer[33]. In this study, we employed the 'wound healing' assay as a preliminary screening tool to evaluate the potential of the compounds to affect cell migration and to identify the most promising candidates for further, more detailed investigation.

Effect in 3D cultures

Experiments in 3D cultures were conducted with hydrazones **7b**, **9e**, **9f** and **10**, which were the most effective at reducing cell viability in monolayer cultures. Spheroids were formed from TNBC, prostate adenocarcinoma, and melanoma cell lines, in combination with human fibroblasts at a 1:1 ratio. These conditions previously established by our group based on the observation that fibroblasts support spheroid formation and enhance their development[34,35]. Additionally, it is well-known that the incorporation of stromal cells into spheroids imparts in vivo-like properties to the model, thereby making it more translatable to real world conditions[36].

At the beginning of the experiment, the spheroids had a diameter of 200–300 μm. The melanoma spheroids grew slightly faster than the other two types, and by the end of the experiment (on the eighth day), their diameter ranged from 350 to 460 μm (Fig. 4, A and **B**). Overall, compounds at a 10 μM concentration exhibited varying effects on spheroid growth. Compound **9e** did not inhibit spheroid growth. In contrast, compound **10** caused the spheroids to become looser, especially the A375 spheroids (Fig. 4A). The most active compound was **9f**, which statistically significantly reduced spheroid size, with some spheroids disintegrating after 6–8 days of incubation. However, this effect was not as pronounced as that observed with the cytotoxic drug doxorubicin. Compound **7b** showed an effect only on the growth of PPC-1 spheroids ($p < 0.05$) (Fig. 4B).

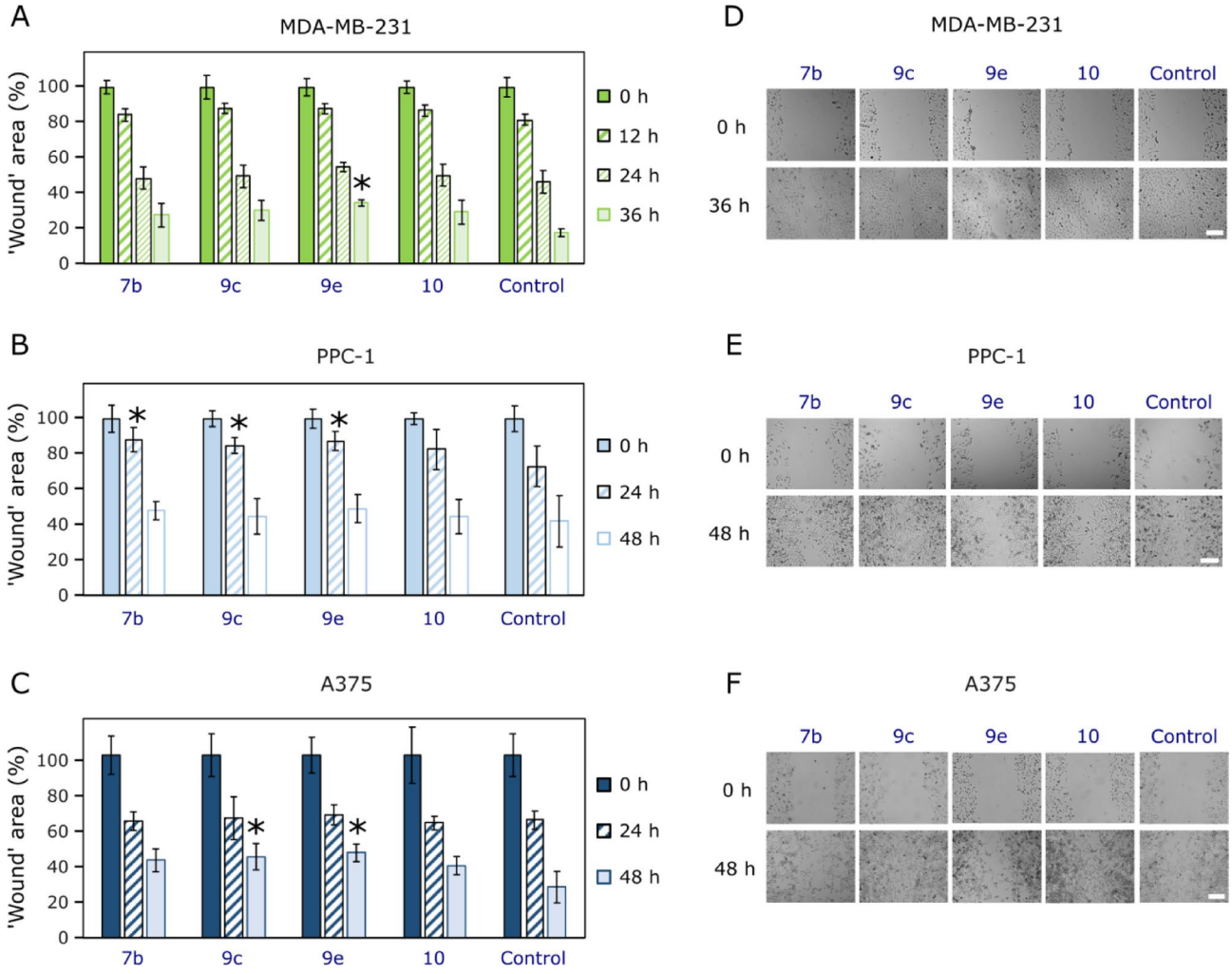


Fig. 3. Effect of compounds **7b**, **9c**, **9e**, and **10** at 10 μM concentration on human triple-negative breast cancer MDA-MB-231 (A), human prostate carcinoma PPC-1r MDA-MB-231 (B), and human malignant melanoma A375 (C) cell migration, $n = 3$. Photos of the 'wound' area in MDA-MB-231 (D), PPC-1 (E), and A375 (F) monolayer at the beginning and the end of the experiment. Asterisk (*) indicates $p < 0.05$. Scale bar indicates 100 μm.

Considering that spheroid size does not necessarily correlate with cell viability, we performed an MTT assay on the final day of the experiment (Fig. 4C). Doxorubicin remained the most active agent in this assay, while compound **9f** was identified as the most cytotoxic compound in 3D cultures. It reduced PPC-1 and A375 spheroid cell viability up to 31.9% and MDA-MB-231 spheroid cell viability up to 53.2% ($p < 0.01$) (Fig. 4C). Notably, compound **7b** also reduced cell viability in all three types of spheroids, despite not having a significant effect on MDA-MB-231 and A375 spheroid growth. This phenomenon further supports previous observations, indicating that assessing compound effects based solely on spheroid size is insufficient[37]. In addition, compound **10** also significantly reduced PPC-1 spheroid cell viability compared to the control ($p < 0.05$).

Overall, compounds exhibited variable anticancer properties in different functional assays. Hydrazone **9f** was identified as the most cytotoxic compound in prostate adenocarcinoma PPC-1 and melanoma A375 cell monolayer and 3D cultures. Although it was less active than doxorubicin, it reduced PPC-1 and A375 cell viability up to 30% after 8 days of incubation. Additionally, **9f** showed the most significant effect among the tested compounds on TNBC cell spheroid growth and viability. In contrast, compound **9e** was not active in 3D cultures and was only moderately cytotoxic in cell monolayer against all cell lines. However, it demonstrated an inhibitory effect on cell migration after different incubation durations. Overall, the selected compounds exhibited greater activity in the melanoma A375 cell line and lower activity against the TNBC MDA-MB-231 cell line.

Conclusions

In conclusion, a series of novel 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylic acid derivatives containing azole and hydrazone fragments was synthesized and investigated for their anticancer activity.

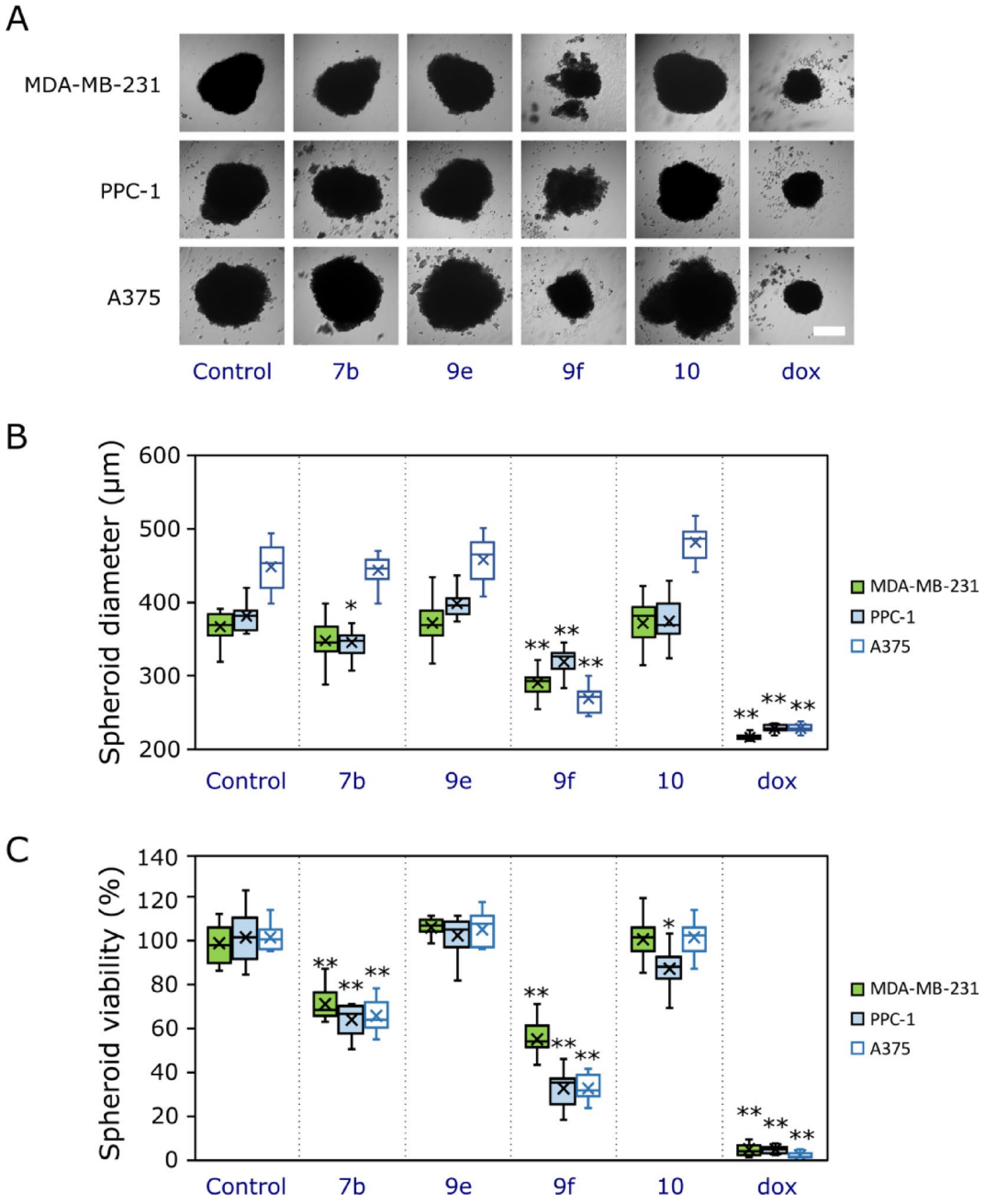


Fig. 4. Effect of the most active compounds **7b**, **9e**, **9f**, **10**, and doxorubicin on 3D cell cultures. (**A**) Photos of human triple-negative breast cancer MDA-MB-231, prostate carcinoma PPC-1 and melanoma A375 tumour spheroids at the end (Day 8) of the experiment (after incubation with 10 µM of compounds). (**B**) MDA-MB-231, PPC-1 and A375 spheroid size at the end of the experiment. (**C**) Viability of cells in MDA-MB-231, PPC-1 and A375 spheroids at the end of the experiment by MTT assay. Asterisks (*) indicate $p < 0.05$ compared to the control (untreated spheroids), (**) indicate $p < 0.01$ compared to the control (untreated spheroids), crosses (×) indicate means; inner dashes indicate medians; whiskers indicate maximum and minimum values. The scale bar indicates 200 µm.

Hydrazone-containing compounds were identified as the most cytotoxic agents against human TNBC MDA-MB-231, prostate carcinoma PPC1 and melanoma A375 cell lines. Generally, compounds **7b**, **9c**, **9e**, **9f** and **10** demonstrated greater activity in the A375 cell line and loweractivity against MDA-MB-231 cells. Among them, compound **9f**, bearing *N*'-(4-methylbenzylidene) moiety, was the most cytotoxic in both prostate adenocarcinoma PPC-1 and melanoma A375 cell monolayer and 3D cultures. It also showed the strongest effect on TNBC cell spheroid growth and viability among the tested compounds. In contrast, compound **9e**, with the *N*'-(4-bromobenzylidene) moiety, was not active in 3D cultures and displayed moderate cytotoxicity in cell monolayer, but it significantly inhibited cell migration after various incubation periods.

Materials and methods

Chemistry

Reagents and solvents were purchased from *Sigma–Aldrich* (St. Louis, MO, USA) and used without further purification. The progress of reactions and the purity of synthesized compounds were monitored by TLC on aluminium plates precoated with *Silica gel with F254 nm* (Merck KGaA, Darmstadt, Germany). Melting points were determined with *a B-540 melting point analyzer* (Büchi Corporation, New Castle, DE, USA) and were uncorrected. NMR spectra were recorded on a *Brucker Avance III (400, 101 MHz) spectrometer* (Bruker BioSpin AG, Fällanden, Switzerland). Chemical shifts were reported in (δ) ppm relative to tetramethylsilane (TMS) with the residual solvent as internal reference (DMSO-d_6, δ = 2.50 ppm for ^{1}H and δ = 39.52 ppm for ^{13}C). Data were reported as follows: chemical shift, multiplicity, coupling constant (Hz), integration, and assignment. IR spectra (ν, cm^{-1}) were recorded on *a Perkin–Elmer Spectrum BX FT–IR spectrometer* (Perkin– Elmer Inc., Waltham, MA, USA) using KBr pellets. Elemental analyses (C, H, N) were conducted using the *Elemental Analyzer CE-440* (Exeter Analytical, Inc., Chelmsford, MA, USA); their results were found to be in good agreement (± 0.3%) with the calculated values.

*1-(2,4-Difluorophenyl)-5-oxopyrrolidine-3-carboxylic acid (**2**)*

A mixture of 2,4-difluoroaniline **(1)** (15 g, 120 mmol), itaconic acid (22.44 g, 170 mmol) and water (30 mL) was refluxed for 22 h, and then cooled. The formed precipitate was filtered off and dissolved in an aqueous 5% sodium hydroxide solution, sodium dithionite (0.8 g) was added, the solution was filtered off, and the filtrate was acidified with hydrochloric acid to pH 1 to give the title compound **2**.

White solid, yield 20.19 g, 73%, m. p. 140–141 °C. IR (KBr): ν 3065 (OH); 1740; 1670 (2x C = O) cm^{-1}.

^{1}H NMR (400 MHz, DMSO–d_6) δ 2.57–2.78 (m, 2 H, $COCH_2$), 3.35–3.55 (m, 1H, CHCO), 3.82–3.99 (m, 2 H, NCH_2), 7.14 (t, *J* = 8.1 Hz, 1H, H_{Ar}), 7.26–7.41 (m,, H_{Ar}), 7.49 (q, *J* = 8.7 Hz, 1H, H_{Ar}), 12.80 (br. s, 1H, COOH) ppm.

^{13}C NMR (101 MHz, DMSO–d_6) δ 33.38 (CH_2CO), 36.27 (CHCO), 51.13 (NCH_2), 104.67, 104.91, 105.18, 111.67, 111.70, 111.89, 111.92, 122.52, 122.56, 122.64, 122.68, 129.10, 129.20, 129.23, 155.60, 155.73, 158.10, 158.23, 159.34, 159.46, 161.79, 161.90 (C_{Ar}), 171.96, 174.08 (2x C = O) ppm. Calcd. for $C_{11}H_9F_2NO_3$, %: C 54.78; H 3.76; N 5.81; Found, %: C 54.63; H 3.73; N 5.80.

General procedure for the Preparation of benzimidazoles 3a–c

A mixture compound **2** (0.7 g, 2.9 mmol) and corresponding *o*-phenylenediamine (5.8 mmol) was refluxed 4 M HCl (8 mL) for 72 h, then cooled and neutralized with aqueous 5% sodium carbonate solution. Formed crystallines products **3a–c** was filtered off, washed with water. Product **3a** was recrystallized from 1,4-dioxane. In the case of products **3b**, **3c**, the obtained compounds was purified by recrystallization from a mixture of 2-propanol and water (1:1, 15 mL). After cooling, the formed crystals were filtered off, washed with diethyl ether to give the title compounds **3b** and **3c**.

*4-(1 H-benzo[d]imidazol-2-yl)-1-(2,4-difluorophenyl)pyrrolidin-2-one (**3a**)*

Yellow solid, yield 0.41 g, 45%, m. p. 162–163 °C. IR (KBr): ν 3018 (NH); 1707 (C = O); 1516 (C = N) cm^{-1}.

^{1}H NMR (400 MHz, DMSO–d_6) δ 3.00–3.23 (m, 2 H, $COCH_2$), 4.19–4.36 (m, 2 H, NCH_2), 4.46 (qu, *J* = 8.1 Hz, 1H, CHCN), 7.19 (t, *J* = 8.5 Hz, 1H, H_{Ar}), 7.33–7.45 (m, 1H, H_{Ar}), 7.46–7.55 (m, 2 H, H_{Ar}), 7.62 (dd, *J* = 15.4, 8.2 Hz, 1H, H_{Ar}), 7.73–7.82 (m, 2 H, H_{Ar}), 15.38 (br. s. 1H, NH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 30.46 (CH_2CO), 35.16 (CHCN), 52.35 (NCH_2), 104.71, 104.98, 105.22, 111.75, 111.97, 112.01, 113.98, 122.24, 122.36, 122.40, 125.19, 129.31, 129.34, 129.41, 129.44, 131.89, 153.46, 155.63, 155.76, 158.14, 158.27, 159.46, 159.58, 161.91, 162.02 (C_{Ar}), 171.00 (C = O) ppm. Calcd. for $C_{17}H_{13}F_2N_3O$, %: C 65.17; H 4.18; N 13.41; Found, %: C 64,98; H 4.13; N 13.29.

*1-(2,4-Difluorophenyl)-4-(6-fluoro-1 H-benzo[d]imidazol-2-yl)pyrrolidin-2-one (**3b**)*

Dark purple solid, yield 0.35 g, 36%, m. p. 108–109 °C. IR (KBr): ν 3108 (NH); 1699 (C = O); 1515 (C = N) cm^{-1}.

^{1}H NMR (400 MHz, DMSO–d_6) δ 2.95 (d, *J* = 7.8 Hz, 2 H, CH_2CO), 4.00–4.21 (m, 3 H, NCH_2 overlaps with CHCN), 6.95–7.08 (m, 1H, H_{Ar}), 7.09–7.23 (m, 1H, H_{Ar}), 7.27–7.62 (m, 4 H, H_{Ar}), 12.59 (s, 1H, NH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 31.89 (CH_2CO), 35.60 (CHCN), 53.24 (NCH_2), 104.04, 104.66, 104.93, 105.17, 109.31, 110.07, 111.65, 111.69, 111.76, 111.91, 119.28, 122.77, 129.09, 129.19, 131.19, 139.43, 156.66 (C_{Ar}), 172.03 (C = O) ppm. Calcd. for $C_{17}H_{12}F_3N_3O$, %: C 61.63; H 3.65; N 12.68; Found, %: C 61.53; H 3.60; N 12.59.

*4-(6-Chloro-1 H-benzo[d]imidazol-2-yl)-1-(2,4-difluorophenyl)pyrrolidin-2-one (**3c**)*

Light brown solid, yield 0.49 g, 49%, m. p. 122–123 °C. IR (KBr): ν 3092 (NH); 1699 (C = O); 1514 (C = N) cm^{-1}.

^{1}H NMR (400 MHz, DMSO–d_6) δ 2.95 (d, *J* = 7.2 Hz, 2 H, CH_2CO), 4.02–4.23 (m, 3 H, NCH_2 overlaps with CHCN), 7.10–7.25 (m, 2 H, H_{Ar}), 7.30–7.45 (m, 1H, H_{Ar}), 7.47–7.67 (m, 3 H, H_{Ar}), 12.67 (s, 1H, NH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 31.89 (CH_2CO), 35.57 (CHCN), 104.66, 104.91, 105.17, 111.65, 111.88, 121.89, 122.59, 122.72, 129.06, 129.09, 129.17, 155.53, 155.66, 156.34, 158.04, 158.16, 159.27, 159.39, 161.72 (C_{Ar}), 172.00 (C = O) ppm. Calcd. for $C_{17}H_{12}ClF_3N_3O$, %: C 58.72; H 3.48; N 12.08; Found, %: C 58.56; H 3.46; N 12.01.

*1-(2,4-Difluorophenyl)-4-(1-ethyl-1 H-benzo[d]imidazol-2-yl)pyrrolidin-2-one (**4**)*

Compound **3a** (0.5 g, 1.6 mmol) was dissolved in dimethylformamide (4 mL). Potassium hydroxide (0.27 g, 4.8 mmol) and potassium carbonate (0.66 g, 4.8 mmol) were added and then the mixture was stirred at 30 °C. After 15 min, ethyl iodide (0.89 g, 5.7 mmol) was added dropwise, and the reaction was carried out at room temperature for 1 h. The volatile fractions were separated under reduced pressure, and then the obtained residue

was diluted with distilled water (15 mL). After cooling, the precipitated crystals were filtered. The desired product **4** was purified by recrystallization from 2-propanol.

Yellow solid, yield 0.37 g, 68%, m. p. 93–94 °C. IR (KBr): ν 1699 (C = O); 1516 (C = N) cm^{-1}.

^{1}H NMR (400 MHz, DMSO–d_6) δ 1.33 (t, J = 7.1 Hz, 3 H, CH_3), 2.88–3.06 (m, 2 H, CH_2CO), 4.11–4.37 (m, 5 H, CH_2CH_3 overlaps with NCH_2 and CHCN), 7.11–7.28 (m, 3 H, H_{Ar}), 7.31–7.46 (m, 1H, H_{Ar}), 7.51–7,66 (m, 3 H, H_{Ar}) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 15.20 (CH_2CH_3), 29.76 (CH_2CH_3), 35.69 (CH_2CO), 37.82 (*C*HCN), 53.25 (NCH_2), 104.65, 104.92, 105.16, 110.15, 111.65, 111.91, 118.85, 121.53, 122.02, 122.77, 129.11, 134.97, 142.08, 154.25, 155.99, 155.72, 158.09, 158.22, 159.29, 161.73, 161.85 (C_{Ar}); 171.94 (C = O) ppm. Calcd. for $C_{19}H_{17}F_2N_3O$, %: C 66.85; H 5.02; N 12.31; Found, %: C 66.77; H 5.01; N 12.26.

*Methyl 1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carboxylate (**5**)*

Ester **5** is obtained by dissolving compound **2** (6.03 g, 25 mmol) in methanol (125 mL) for 2 h at the boiling temperature of the mixture and using 0.5 mL of concentrated sulfuric acid as a catalyst. At the end of the reaction, the solvent is distilled under pressure. The residue is neutralized using 5% sodium carbonate solution (150 mL). The formed crystals are filtered and washed with water. Purified by recrystallization from a mixture of methanol and water (1:1).

Yellowish, yield 4.85 g, 76%, m. p. 94–95 °C. IR (KBr): ν 1741; 1699 (C = O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ 2.60–2.81 (m, 2 H, CH_2CO), 3.46–3.58 (m, 1H, CHCO), 3.68 (s, 3 H, CCH_3), 3.82–3.99 (m, 2 H, NCH_2), 7.04–7.19 (m, 1H, H_{Ar}), 7.29–7.43 (m, 1H, H_{Ar}), 7.49 (dd, J = 15.1, 8.7 Hz, 1H, H_{Ar}) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 33,86 (CH_2CO), 36.66 (*C*HCO), 51.50 (NCH_2), 52.82 (CH_3), 105.29, 105.53, 105.80, 112.29, 112.32, 112.51, 112.55, 123.06, 123.19, 129.73, 129.86, 156.21, 156.34, 158.71, 158.84, 159.98, 160.10, 162.43, 162.54 (C_{Ar}), 172.27, 173.63 (2x C = O) ppm. Calcd. for $C_{12}H_{11}F_2NO_3$, %: C 56.47; H 4.34; N 5.49; Found, %: C 56.40; H 4.31; N 5.45.

*1-(2,4-Difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**6**)*

Compound **5** (5.10 g, 20 mmol) was dissolved in 2-propanol (25 mL), hydrazine monohydrate (2.00 g, 40 mmol) was added dropwise. Mixture of reaction was refluxed for 8 h, then cooled down. The obtained crystalline solid was filtered off, washed with 2-propanol to give the title compound **6.**

Yellowish solid, yield 3.16 g, 62%, m. p. 126–127 °C (from 2-propanol). IR (KBr): ν 3283, 3192 ($NH + NH_2$); 1694; 1637 (2x C = O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ 2.56–2.64 (m, 2 H, CH_2CO), 3.19–3.27 (m, 1H, CHCO), 3.75 (t, J = 7.9 Hz, 1H, NCH_2), 3.84 (t, J = 8.4 Hz, 1H, NCH_2), 4.29 (s, 2 H, $NHNH_2$), 7.03–7.18 (m, 1H, H_{Ar}), 7.29–7.42 (m, 1H, H_{Ar}), 7.49 (dd, J = 15.1, 8.7 Hz, 1H, H_{Ar}), 9.28 (s, 1H, NH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 33.88 (CH_2CO), 35.37 (*C*HCO), 51.79 (NCH_2), 104.65, 104.89, 105.16, 111.69, 111.91, 122.56, 122.60, 122.68, 122.72, 129.18, 129.21, 129.28, 129.31, 155.59, 155.72, 158.09, 158.22, 159.30, 159.41, 161.75, 161.86 (C_{Ar}), 171.21, 171.17 (2x C = O) ppm. Calcd. for $C_{11}H_{11}F_2N_3O_2$, %: C 51.77; H 4.34; N 16.46; Found, %: C 51.64; H 4.31; N 16.39.

General procedure for the Preparation of hydrazones 7a, B and 9a–h

To a solution of hydrazide **6** (0.3 g 1 mmol) in 2-propanol (7 mL), the corresponding aromatic or heterocyclic aldehyde (1.2 mmol) was added and the mixture was heated at reflux for 2 h. After completion of the reaction, the mixture was cooled, and the formed precipitate was filtered off and washed with 2-propanol to give the corresponding compounds **7a**, **b** and **9a–h**.

*1-(2,4-Difluorophenyl)-5-oxo-N'-(thiophen-2-ylmethylene)pyrrolidine-3-carbohydrazide (**7a**)*

Orange solid, yield 0.250 g, 72%, m. p. 119–120 °C (from 2-propanol). IR (KBr): ν 3240 (NH); 1703; 1662 (2x C = O); 1509 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.63–2,83 (m, 2 H, CH_2CO), 3.35–3.44, 3.81–4.07 (2 m, 3 H, CHCO, NCH_2), 7.07–7.20 (m, 2 H, H_{Ar}), 7.33–7.58 (m, 3 H, H_{Ar}), 7.64 (dd, J = 14.7, 4.9 Hz, 1H, H_{Ar}), 8.20, 8.43 (2s, 1H, NHNC*H*), 11.56 (s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 33.49, 34.42 (CH_2CO), 34.83, 36.67 (*C*HCO), 51.83, 52.24 (NCH_2), 105.28, 105.55, 105.79, 112.31, 112.50, 112.54, 123.17, 123.29, 123.32, 128.48, 128.59, 129.10, 129.64, 129.85, 129.95, 131.04, 131.71, 139.28, 139.50, 139.50, 139.55, 142.85, 156.24, 156.36, 158.74, 158.87, 159.93, 160.04, 162.38, 162.50 (C_{Ar}), 168.89 (C = N), 172.73, 173.49 (2x C = O) ppm. Calcd. for $C_{16}H_{13}F_2N_3O_2S$, %: C 55.01; H 3.75; N 12.03; Found, %: C 54.94; H 3.71; N 11.97.

*1-(2,4-Difluorophenyl)-N'-((5-nitrothiophen-2-yl)methylene)-5-oxopyrrolidine-3-carbohydrazide (**7b**)*

Orange solid, yield 0.39 g, 96%, m. p. 197–198 °C (from 2-propanol). IR (KBr): ν 3200 (NH); 1697; 1677 (2x C = O); 1520 (C = N); 1175 cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.80 (m, 2 H, CH_2CO), 3.38–3.48, 3.82–4.13 (2 m, 3 H, CHCO, NCH_2), 7.03–7.21 (m, 1H, H_{Ar}), 7.26–7.43 (m, 1H, H_{Ar}), 7.46–7.60 (m, 2 H, H_{Ar}), 8.06–8.14 (m, 1H, H_{Ar}), 8.19, 8.47 (2s, 1H, NHNC*H*), 11.98 (s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.91, 33.68 (CH_2CO), 34.00, 36.11 (*C*HCO), 51.05, 51.41 (NCH_2), 104.91, 105.16, 111.67, 111.86, 111.90, 111.93, 122.46, 122.50, 122.54, 122.58, 122.62, 122.67, 129.23, 129.31, 129.34, 129.37, 129.79, 130.46, 130.61, 136.92, 140.61, 146.49, 146.51, 150.55, 150.88, 155.60, 155.74, 158.11, 159.31, 159.43, 161.76, 161.88 (C_{Ar}), 168.97 (C = N), 171.99, 173.60 (2x C = O) ppm. Calcd. for $C_{16}H_{12}F_2N_4O_2S$, %: C 48.73; H 3.07; N 14.21; Found, %: C 48.64; H 3.03; N 14.20.

*N'-Benzylidene-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**9a**)*

Brownish solid, yield 0.27 g, 78%, m. p. 159–160 °C (from 2-propanol). IR (KBr) ν 3039 (NH); 1703; 1676 (2x C = O); 1515 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.84 (m, 2 H, CH_2CO), 3.36–3.46, 3.83–4.21 (2 m, 3 H, CHCO, NCH_2), 7.03–7.21 (m, 1H, H_{Ar}), 7.32–7.48 (m, 4 H, H_{Ar}), 7.49–7.58 (m,

1H, H_{Ar}), 7.63–7.75 (m, 2 H, H_{Ar}), 8.03, 8.22 (2s, 1H, NHNC*H*), 11.58, 11.62 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.96, 33.80 (*C*H_2CO), 34.04, 36.06 (*C*HCO), 51.21, 51.63 (NCH_2), 104.64, 104.88, 104.90, 105.15, 111.62, 111.65, 111.84, 111.88, 111.91, 122.51, 122.58, 122.62, 122.64, 122.70, 122.74, 126.87, 127.09, 128.84, 129.20, 129.28, 129.91, 130.12, 134.10, 134.13, 143.68, 147.04, 155.61, 155.73, 158.11, 158.24, 159.29, 159.40, 159.42, 161.73, 161.75, 161.85 (C_{Ar}), 168.40 (C = N), 172.13, 173.31 (2x C = O) ppm. Calcd. for $C_{18}H_{15}F_2N_3O_2$, %: C 62.97; H 4.40; N 12.24; Found, %: C 62.91; H 4.35; N 12.19.

*1-(2,4-Difluorophenyl)-N'-(4-fluorobenzylidene)-5-oxopyrrolidine-3-carbohydrazide (**9b**)*
Yellowish solid, yield 0.29 g, 78%, m. p. 167–168 °C (from 2-propanol). IR (KBr) ν 3070 (NH); 1707; 1668 (2x C = O); 1514 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.83 (m, 2 H, CH_2CO), 3.36–3.46, 3.82–4.20 (2 m, 3 H, CHCO, NCH_2), 7.05–7.20 (m, 1H, H_{Ar}), 7.21–7.32 (m, 2 H, H_{Ar}), 7.31–7.43 (m, 1H, H_{Ar}), 7.53 (dd, *J* = 14.5, 7.9 Hz, 1H, H_{Ar}), 7.69–7.81 (m, 2 H, H_{Ar}), 8.02, 8.21 (2s, 1H, NHNC*H*), 11.58, 11.63 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.96, 33.79 (*C*H_2CO), 34.00, 36.04 (*C*HCO), 51.20, 51.62 (NCH_2), 104.64, 104.90, 105.15, 111.62, 111.65, 111.84, 111.87, 111.91, 115.77, 115.99, 122.53, 122.58, 122.63, 122.67, 122.70, 122.74, 129.02, 129.10, 129.15, 129.23, 129.29, 129.31, 130.71, 130.74, 142.55, 145.92, 155.60, 155.73, 158.10, 158.23, 159.26, 159.29, 159.40, 161.85, 161.91, 164.22 (C_{Ar}), 168.41 (C = N), 172.11, 173.31 (2x C = O) ppm. Calcd. for $C_{18}H_{14}F_3N_3O_2$, %: C 59.83; H 3.91; N 11.63; Found, %: C 59.78; H 3.82; N 11.59.

*N'-(4-Chlorobenzylidene)-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**9c**)*
Brownish solid, yield 0.32 g, 84%, m. p. 177–178 °C (from 2-propanol). IR (KBr) ν 3216 (NH); 1682 (2x C = O); 1514 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.79 (m, 2 H, CH_2CO), 3.36–3.46, 3.83–4.21 (2 m, 3 H, CHCO, NCH_2), 7.10–7.19 (m, 1H, H_{Ar}), 7.33–7.43 (m, 1H, H_{Ar}), 7.48–7.58 (m, 3 H, H_{Ar}), 7.72 (d, *J* = 8.2 Hz, 2 H, H_{Ar}), 8.02, 8.20 (2s, 1H, NHNC*H*), 11.64, 11.69 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.97, 33.76 (*C*H_2CO), 33.98, 36.07 (*C*HCO), 51.15, 51.59 (NCH_2), 104.64, 104.91, 105.15, 111.66, 111.88, 122.57, 128.54, 128.72, 128.91, 129.28, 133.05, 133.09, 134.33, 134.56, 142.42, 145.72, 155.50, 155.59, 155.72, 158.10, 158.22, 159.28, 159.42, 161.73, 161.85 (C_{Ar}), 168.49 (C = N), 172.07, 173.39 (2x C = O) ppm. Calcd. for $C_{18}H_{14}ClF_2N_3O_2$, %: C 57.23; H 3.74; N 11.12; Found, %: C 57.20; H 3.71; N 11.03.

*N'-(2,4-Difluorobenzylidene)-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**9d**)*
White solid, yield 0.29 g, 76%, m. p. 175–176 °C (from 2-propanol). IR (KBr) ν 3185 (NH); 1708; 1675 (2x C = O); 1512 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.81 (m, 2 H, CH_2CO), 3.34–3.45, 3.83–4.20 (2 m, 3 H, CHCO, NCH_2), 7.09–7.23 (m, 2 H, H_{Ar}), 7.30–7.43 (m, 2 H, H_{Ar}), 7.47–7.58 (m, 1H, H_{Ar}), 7.88–8.02 (m, 1H, H_{Ar}), 8.17, 8.38 (2s, 1H, NHNC*H*), 11.68, 11.75 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.93, 33.73 (*C*H_2CO), 33.99, 36.10 (*C*HCO), 51.12, 51.52 (NCH_2), 104.24, 104.49, 104.64, 104.91, 105.15, 111.66, 111.88, 112.51, 118.46, 118.50, 118.60, 122.56, 128.03, 129.18, 129.28, 135.89, 139.06, 155.61, 158.11, 158.24, 159.48, 159.60, 162.12, 164.42 (C_{Ar}), 168.49 (C = N), 172.07, 173.40 (2x C = O) ppm. Calcd. for $C_{18}H_{13}F_4N_3O_2$, %: C 57.00; H 3.45; N 11.08; Found, %: C 56.93; H 3.41; N 10.94.

*N'-(4-Bromobenzylidene)-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**9e**)*
White solid, yield 0.39 g, 92%, m. p. 179–180 °C (from 2-propanol). IR (KBr) ν 3218 (NH); 1708; 1681 (2x C = O); 1513 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.79 (m, 2 H, CH_2CO), 3.36–3.46, 3.82–4.20 (2 m, 3 H, CHCO, NCH_2), 7.10–7.19 (m, 1H, H_{Ar}), 7.33–7.42 (m, 1H, H_{Ar}), 7.48–7.58 (m, 1H, H_{Ar}), 7.58–7.69 (m, 4 H, H_{Ar}), 8.00, 8.19 (2s, 1H, NHNC*H*), 11.64, 11.69 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.97, 33.76 (*C*H_2CO), 33.97, 36.04 (*C*HCO), 51.15, 51.56 (NCH_2), 104.65, 104.91, 105.15, 111.66, 111.88, 122.69, 123.10, 123.36, 128.77, 125.95, 129.28, 131.82, 133.39, 142.52, 145.80, 155.60, 155.72, 158.10, 158.22, 159.29, 159.40, 161.74, 161.87 (C_{Ar}), 168.49 (C = N), 172.07, 173.40 (2x C = O) ppm. Calcd. for $C_{18}H_{14}BrF_2N_3O_2$, %: C 51.20; H 3.34; N 9.95; Found, %: C 51.15; H 3.28; N 9.88.

*1-(2,4-Difluorophenyl)-N'-(4-methylbenzylidene)-5-oxopyrrolidine-3-carbohydrazide (**9f**)*
Yellowish solid, yield 0.25 g, 70%, m. p. 138–139 °C (from 2-propanol). IR (KBr) ν 3224 (NH); 1685 (2x C = O); 1513 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.32 (s, 3 H, CCH_3), 2.64–2.83 (m, 2 H, CH_2CO), 3.35–3.45, 3.82–4.19 (2 m, 3 H, CHCO, NCH_2), 7.09–7.19 (m, 1H, H_{Ar}), 7.21–7.29 (m, 2 H, H_{Ar}), 7.33–7.43 (m, 1H, H_{Ar}), 7.48–7.63 (m, 3 H, H_{Ar}), 7.99, 8.17 (2s, 1H, NHNC*H*), 11.51, 11.55 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 21.01 (*C*CH_3), 32.97, 33.80 (*C*H_2CO), 34.04, 36.05 (*C*HCO), 51.23, 51.65 (NCH_2), 104.90, 105.15, 111.62, 111.65, 111.67, 111.84, 111.87, 111.91, 126.84, 127.07, 129.18, 129.21, 129.28, 129.30, 129.43, 131.40, 139.70, 139.96, 143.76, 147.07, 155.60, 155.73, 158.10, 158.23, 159.28, 159.40, 161.72, 161.84 (C_{Ar}), 168.27 (C = N), 172.14, 173.19 (2x C = O) ppm. Calcd. for $C_{19}H_{17}F_2N_3O_2$, %: C 63.86; H 4.80; N 11.76; Found, %: C 63.80; H 4.74; N 11.72.

*1-(2,4-Difluorophenyl)-N'-(4-(dimethylamino)benzylidene)-5-oxopyrrolidine-3-carbohydrazide (**9 g**)*
Light yellow solid, yield 0.30 g, 77%, m. p. 163–164 °C (from 2-propanol). IR (KBr) ν 3067 (NH); 1703; 1671 (2x C = O); 1513 (C = N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–3.02 (m, 8 H, CH_2CO overlaps with N(CH_3)$_2$), 3.30–3.43, 3.80–4.16 (2 m, 3 H, CHCO, NCH_2), 6.56–6.80 (m, 2 H, H_{Ar}), 7.09–7.56 (m, 5 H, H_{Ar}), 7.89, 8.06 (2s, 1H, NHNC*H*), 11.29, 11.31 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 25.49 (N(CH_3)$_2$), 32.93, 33.88 (*C*H_2CO), 34.07, 36.03 (*C*HCO), 51.34, 51.74 (NCH_2), 104.63, 104.90, 105.14, 111.62, 111.65, 111.77, 111.82, 111.87, 111.90, 121.34, 121.47, 122.65, 128.11, 128.42, 129.19, 129.30, 144.46, 147.83, 151.37, 151.53, 155.60, 155.73, 158.10, 158.23, 159.27, 159.39, 161.86 (C_{Ar}), 167.73 (C = N), 172.23, 172.64 (2x C = O) ppm. Calcd. for $C_{20}H_{20}F_2N_4O_2$, %: C 62.17; H 5.22; N 14.50; Found, %: C 62.08; H 5.19; N 14.44.

*1-(2,4-Difluorophenyl)-N'-(4-nitrobenzylidene)-5-oxopyrrolidine-3-carbohydrazide (**9 h**)*
Bright yellow solid, yield 0.35 g, 89%, m. p. 204–205 °C (from 2-propanol). IR (KBr) ν 3087 (NH); 1701; 1683 (2x C = O); 1514 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.64–2.83 (m, 2 H, CH_2CO), 3.40–3.51, 3.84–4.25 (2 m, 3 H, CHCO, NCH_2), 7.10–7.20 (m, 1H, H_{Ar}), 7.31–7.43 (m, 1H, H_{Ar}), 7.47–7.59 (m, 1H, H_{Ar}), 7.97 (d, *J* = 8.5 Hz, 2 H, H_{Ar}), 8.09–8.34 (m, 3 H, H_{Ar} overlaps with NHNC*H*), 11.88 (s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.98, 33.72 (*C*H_2CO), 34.00, 36.10 (*C*HCO), 51.07, 51.52 (NCH_2), 104.66, 104.92, 105.16, 111.66, 111.89, 122.58, 122.67, 124.02, 124.12, 127.85, 128.02, 129.13, 129.17, 129.23, 129.26, 140.39, 140.48, 141.38, 144.58, 147.72, 147.89, 154.43, 155.73, 156.52, 158.10, 158.23, 159.30, 159.42, 159.48, 161.77, 161.82, 161.89 (C_{Ar}), 168.88 (C = N), 172.00, 173.75 (2x C = O) ppm. Calcd. for $C_{18}H_{14}F_2N_4O_4$, %: C 55.67; H 3.63; N 14.43; Found, %: C 55.60; H 3.59; N 14.39.

General method of the Preparation of hydrazones 8a, B

A mixture of hydrazide **6** (0.30 g 1 mmol), acetone (3 mL in order to synthesize product **8a**) or methylethylketone (3 mL to obtain product **8b**) and 1 drop of acetic acid was heated at reflux for 1 h, then cooled and the formed crystalline solid was filtered off, washed with hot hexane to give the title compound **8a** and **8b**.

*1-(2,4-Difluorophenyl)-5-oxo-N'-(propan-2-ylidene)pyrrolidine-3-carbohydrazide (**8a**)*
Brownish solid, yield 0.25 g, 85%, m. p. 124–125 °C (from 2-propanol: water 1:1). IR (KBr) ν 3217 (NH); 1701; 1664 (2x C = O); 1514 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 1.81–1.97 (m, 6 H, $C(CH_3)_2$), 2.57–2.76 (m, 2 H, CH_2CO), 3.44–3.54, 3.71–4.02 (2 m, 3 H, CHCO, NCH_2), 7.02–7.21 (m, 1H, H_{Ar}), 7.26–7.42 (m, 1H, H_{Ar}), 7.51 (dd, *J* = 14.6, 6.9 Hz, 1H, H_{Ar}), 10.19, 10.31 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 17.16, 17.31 (*C*CH_3), 24.96, 25.23 (*C*CH_3), 32.94, 33.93. 34.00 (*C*H_2CO), 34.24, 35.67 (*C*HCO), 51.40, 51.99 (NCH_2), 104.88, 111.65, 111.87, 122.72, 129.25, 151.29, 156.24, 158.09, 159.41, 161.72 (C_{Ar}), 168.23 (C = N), 172.20, 173.36 (2x C = O) ppm. Calcd. for $C_{14}H_{15}F_2N_3O_2$, %: C 56.95; H 5.12; N 14.23; Found, %: C 56.87; H 5.10; N 14.18.

*N'-(Butan-2-ylidene)-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**8b**)*
Orange solid, yield 0.20 g, 65%, m. p. 92–93 °C (from 2-propanol: water 1:1). IR (KBr) ν 3209 (NH); 1713; 1699 (2x C = O); 1513 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 0.93–1.07 (m, 3 H, CH_2CH_3), 1.78–1.95 (m, 3 H, CCH_3), 2.16–2.34 (m, 2 H, CH_2CH_3), 2.57–2.79 (m, 2 H, CH_2CO), 3.45–3.55, 3.72–4.05 (2 m, 3 H, CHCO, NCH_2), 7.02–7.20 (m, 1H, H_{Ar}), 7.27–7.43 (m, 1H, H_{Ar}), 7.51 (dd, *J* = 15.2, 7.9 Hz, 1H, H_{Ar}), 10.17, 10.34 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 9.76, 10.33, 10.76 (CH_2CH_3), 16.04, 16.08 (CCH_3), 22.16, 22.54, 22.99, 23.44 (*C*H_2CH_3), 31.43, 31.54, 32.87, 32.94, 33.97 (*C*H_2CO), 34.24, 35.65, 35.72 (*C*HCO), 51.32, 51.34, 51.98 (NCH_2), 104.63, 104.88, 105.14, 111.65, 111.87, 122.60, 122.64, 122.72, 129.18, 129.21, 129.24, 129.31, 154.57, 155.39, 155.59, 155.72, 158.09, 158.22, 159.72, 160.03, 161.72, 161.83 (C_{Ar}), 168.31 (C = N), 172.29, 173.46 (2x C = O) ppm. Calcd. for $C_{15}H_{17}F_2N_3O_2$, %: C 58.25; H 5.54; N 13.59; Found, %: C 58.12; H 5.49; N 13.55.

*N'-[1-(4-Aminophenyl)ethylidene]-1-(2,4-difluorophenyl)-5-oxopyrrolidine-3-carbohydrazide (**8c**)*
A mixture of hydrazide **6** (0.2 g, 0.78 mmol), 4'-aminoacetophenone (0.11 g, 0.82 mmol) and 3 drops of acetic acid in 2-propanol (5 mL) was heated at reflux for 5 h, later the reaction mixture was cooled and formed solid was filtered off, washed with hot hexane and dried to give the title compound **8c**.

Yellowish solid, yield 0.16 g, 56%, m. p. 191–192 °C (from 1,4-dioxane). IR (KBr) ν 3498; 3388 (NH_2, NH); 1691; 1670 (2x C = O); 1513 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ (Z/E 65/35): 2.16 (d, *J* = 8.4 Hz, 3 H, CCH_3), 2.64–2.84 (m, 2 H, CH_2CO), 3.53–3.64, 3.79–4.21 (2 m, 3 H, CHCO, NCH_2), 5.38–5.51 (m, 2 H, CNH_2), 6.55 (d, *J* = 8.3 Hz, 2 H, H_{Ar}), 7.05–7.22 (m, 1H, H_{Ar}), 7.38 (dd, *J* = 17.1, 8.2 Hz, 1H, H_{Ar}), 7.43–7.63 (m, 3 H, H_{Ar}), 10.34, 10.49 (2s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 13.26, 13.79 (CCH_3), 33.02, 34.05 (CH_2CO), 34.33, 35.88 (*C*HCO), 51.50, 52.07 (NCH_2), 104.64, 104.90, 105.15, 111.62, 111.65, 111.84, 111.88, 113.12, 113.26, 122.62, 122.66, 122.73, 122.77, 125.04, 125.20, 127.23, 127.61, 129.22, 129.25, 129.32, 129.34, 149.08, 150.05, 150.28, 153.68, 155.62, 155.73, 158.10, 158.25, 159.27, 159.38, 161.71, 161.83 (C_{Ar}), 168.35 (C = N), 172.35, 173.63 (2x C = O) ppm. Calcd. for $C_{19}H_{18}F_2N_4O_2$, %: C 61.28; H 4.87; N 15.05; Found, %: C 61.04; H 4.86; N 14.98.

*N'-(4-Bromobenzylidene)-1-(2,4-difluorophenyl)-N-ethyl-5-oxopyrrolidine-3-carbohydrazide (**10**)*
Compound **9e** (0.25 g, 0.6 mmol) was dissolved in dimethylformamide (2 mL). Pottasium hydroxide (0.10 g, 1.8 mmol) and pottasium carbonate (0.25 g, 1.8 mmol) were added and then the mixture was stirred at 30 °C. After 15 min, ethyl iodide (0.34 g, 2.2 mmol) was added dropwise, and the reaction was carried out at the room temperature for 1 h. The volatile fractions were separated under reduced pressure and then obtained residue was diluted with distilled water (10 mL). After cooling, the precipitated crystals were filtered. The desired product **10** was purified by recrystallization from 1,4-dioxane: water (2:1).

White solid, yield 0.14 g, 54%, m. p. 182–183 °C. IR (KBr) ν 1698; 1673 (2x C = O); 1515 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ 1.09 (t, *J* = 6.9 Hz, 3 H, CH_2CH_3), 2.75 (d, *J* = 8.4 Hz, 2 H, CH_2CO), 3.80–4.39 (3 m, 5 H, CH_2CH_3, CHCO, NCH_2), 7.05–7.21 (m, 1H, H_{Ar}), 7.27–7.42 (m, 1H, H_{Ar}), 7.52 (dd, *J* = 15.2, 8.5 Hz, 1H, H_{Ar}), 7.65 (d, *J* = 8.0 Hz, 2 H, H_{Ar}), 7.75 (d, *J* = 8.1 Hz, 2 H, H_{Ar}), 8.08 (s, 1H, NNCH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 10.84 (CH_2CH_3), 33.45 (CH_2CO), 34.52 (CH_2CH_3), 35.30 (CHCO), 51.63 (NCH_2), 104.65, 104.92, 105.16, 111.63, 111.88, 122.92, 128.95, 129.23, 131.81, 134.10, 139.33 (C_{Ar}), 172.11, 172.60 (2x C = O) ppm. Calcd. for $C_{20}H_{18}F_2N_3O_2$, %: C 53.25; H 4.05; N 9.33; Found, %: C 53.26; H 4.03; N 9.28.

*1-(2,4-Difluorophenyl)-N-(2,5-dimethyl-1 H-pyrrol-1-yl)-5-oxopyrrolidine-3-carboxamide (**11**)*
A mixture of hydrazide **6** (0.50 g, 2 mmol), hexane-2,5-dione (0.42 mL, 3.6 mmol), 2-propanol (9 mL) and glacial acetic acid (3 drops) was refluxed for 2 h, then cooled. The formed precipitate was filtered off, recrystalized from 2-propanol to give the title compound **11.**

Yellowish solid, yield 0.45 g, 68%, m. p. 180–181 °C. IR (KBr) ν 3313 (NH); 1711; 1682 (2x C = O) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ 1.91–2.11 (m, 6 H, 2x CH_3), 2.65–2.86 (m, 2 H, CH_2CO), 3.47–3.60 (m, 1H, CHCO), 3.84–3.92, 3.98–4.09 (2 m, 2 H, NCH_2), 5.65 (s, 2 H, 2x NCCH), 7.16 (t, *J* = 8.3 Hz, 1H, H_{Ar}), 7.39 (t, *J* = 10.0 Hz, 1H, H_{Ar}), 7.52 (dd, *J* = 15.4, 8.0 Hz, 1H, H_{Ar}), 10.89 (s, 1H, CONH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 10.88, 10.94 (2x CCH_3), 33.57 (CH_2CO), 35.26 (*C*HCO), 51.60, 51.62 (NCH_2), 103.10, 104.67, 104.92, 104.94, 105.18, 111.69, 111.73, 111.92, 111.95, 122.42, 122.46, 122.55, 122.58, 126.73, 126.76, 129.27, 129.30, 129.37, 129.40, 155.65, 155.78, 158.15, 158.28, 159.37, 159.48, 161.82, 161.93 (C_{Ar}), 171.58, 171.86 (2x C = O) ppm. Calcd. for $C_{17}H_{17}F_2N_3O_2$, %: C 61.26; H 5.14; N 12.61; Found, %: C 61.17; H 5.12; N 12.55.

*1-(2,4-Difluorophenyl)-4-(3,5-dimethyl-1 H-pyrazole-1-carbonyl)pyrrolidin-2-one (**12**)*
A mixture of hydrazide **6** (0.40 g, 1.6 mmol), 2,4-pentanedione (0.25 mL, 2.4 mmol), 2-propanol (7 mL) and hydrochloric acid (3 drops) was heated at reflux for 2 h, then cooled. The formed precipitate was filtered off, recrystalized from 2-propanol, dried to give the title compound **12.**

Orange solid, yield 0.20 g, 39%, m. p. 110–111 °C. IR (KBr) ν 1724; 1699 (2x C = O); 1517 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ 2.19 (s, 3 H, CCH_3), 2.50 (s, 3 H, CCH_3 overlaps with DMSO–d_6), 2.81 (d, *J* = 8.2 Hz, 2 H, CH_2CO), 3.88–3.96, 4.05–4.17 (2 m, 2 H, NCH_2), 4.47–4.58 (m, 1H, CHCO), 6.23 (s, 1H, NCCH), 7.07–7.19 (m, 1H, H_{Ar}), 7.29–7.43 (m, 1H, H_{Ar}), 7.53 (dd, *J* = 15.5, 8.1 Hz, 1H, H_{Ar}) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 13.11, 13.62 (2x CCH_3), 32.81 (CH_2CO), 36.27 (*C*HCO), 50.89 (NCH_2), 104.22, 104.46, 104.73, 111.15, 111.22, 111.44, 111.48, 121.96, 122.00, 122.09, 122.12, 128.81, 128.84, 128.91, 128.94, 143.45, 151.73, 155.20, 155.33, 157.70, 157.83, 158.93, 159.05, 161.38, 161.50 (C_{Ar}), 171.21, 171.93 (2x C = O) ppm. Calcd. for $C_{16}H_{15}F_2N_3O_2$, %: C 60.18; H 4.74; N 13.16; Found, %: C 60.11; H 4.72; N 13.13.

General method of the Preparation of carbo(thio)amides 13, 15
Hydrazide **6** (0.50 g, 2 mmol) is dissolved in methanol (7 mL), then corresponding reagent is added dropwise – phenyl isocyanate (0.67 g, 5.6 mmol) to obtain compound **13**, phenyl isothiocyanate (0.27 g, 2 mmol) in order to synthesize compound **15**. Reactions were heated at reflux for 3 h, respectively. Reaction mixture was dissolved with water: methanol (1:1), the formed precipitate was filtered off, recrystalized from 1,4-dioxane and dried to give the title compounds **13** and **15.**

*2-[1-(2,4-Difluorophenyl)-5-oxopyrrolidine-3-carbonyl]-N-phenylhydrazine-1-carboxamide (**13**)*
White solid, yield 0.61 g, 81%, m. p. 174–175 °C. IR (KBr) ν 3319; 3269; 3146 (3x NH); 1724; 1682; 1663 (3x C = O) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ 2.58–2.78 (m, 2 H, CH_2CO), 3.34–3.44 (m, 1H, CHCO), 3.77–3.99 (m, 2 H, NCH_2), 6.87-7.00 (m, 1H, H_{Ar}), 7.09–7.19 (m, 1H, H_{Ar}), 7.20–7.29 (m, 2 H, H_{Ar}), 7.33–7.55 (m, 4 H, H_{Ar}), 8.11, 8.78, 9.92 (3s, 3 H, 3x NH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 33.66 (CH_2CO), 35.02 (*C*HCO), 51.58 (NCH_2), 104.67, 104.94, 105.18, 111.67, 111.90, 118.51, 121.97, 122.68, 128.68, 129.18, 129.28, 139.56, 155.25, 155.59, 155.71, 158.09, 158.22, 159.30, 159.42, 161.75, 161.87 (C_{Ar}), 172.01, 172.22 (3x C = O) ppm. Calcd. for $C_{18}H_{16}F_2N_4O_3$, %: C 57.75; H 4.31; N 14.97; Found, %: C 57.60; H 4.25; N 14.92.

*2-[1-(2,4-Difluorophenyl)-5-oxopyrrolidine-3-carbonyl]-N-phenylhydrazine-1-carbothioamide (**15**)*
White solid, yield 0.48 g, 61%, m. p. 110–111 °C. IR (KBr) ν 3568; 3271; 3169 (3x NH); 1696; 1668 (2x C = O); 1209 (C = S) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ 2.62–2.79 (m, 2 H, CH_2CO), 3.34–3.46 (m, 1H, CHCO), 3.80–4.00 (m, 2 H, NCH_2), 7.10–7.22 (m, 2 H, H_{Ar}), 7.27–7.58 (m, 6 H, H_{Ar}), 9.58, 9.85, 10.18 (3s, 3 H, C = ONH, NHC = S, NHC) ppm.

^{13}C NMR (101 MHz, DMSO–d_6) δ 33.63 (CH_2CO), 35.23 (*C*HCO), 51.50 (NCH_2), 104.95, 105.19, 111.73, 111.95, 116.79, 122.57, 122.70, 125.26, 125.31, 125.37, 125.42, 125.69, 125.64, 126.31, 126.38, 126.63, 128.20, 129.17, 129.28, 139.05, 155.58, 155.70, 158.08, 158.20, 159.30, 159.42, 161.75, 161.86, 161.89 (C_{Ar}), 172.04 (2x C = O), 181.16 (C = S) ppm. Calcd. for $C_{18}H_{16}F_2N_4O_2S$, %: C 55.38; H 4.13; N 14.35; Found, %: C 55.32; H 4.10; N 14.29.

*4-[(2,4-Difluorophenyl)amino]-3-(5-oxo-4-phenyl-4,5-dihydro-1 H-1,2,4-triazol-3-yl)butanoic acid (**14**)*
Compound **13** (0.35 g, 0.9 mmol) was dissolved in 4% NaOH solution (7 mL). Reaction was heated at reflux for 19 h, later sodium hydroxide was neutralized using diluted hydrochloric acid (water: HCl, 1:1) until pH 6 was reached. Formed crystals were filtered, dried and recrystalized using 1,4-dioxane.

Light brown solid, yield 0.16 g, 43%, m. p. 168–169 °C. IR (KBr) ν 3444; 3312 (2x NH); 3074 (OH); 1716; 1692 (2x C = O); 1519 (C = N) cm^{-1}. ^1H NMR (400 MHz, DMSO–d_6) δ 2.59–2.78 (m, 2 H, CH_2COOH), 2.79–3.24 (m, 3 H, CNCH overlaps with $NHCH_2$), 5.60 (s, 1H, CH_2NH), 5.85 (dd, *J* = 14.8, 9.3 Hz, 1H, H_{Ar}), 6.39–6.58 (m, 1H, H_{Ar}), 6.92–7.02 (m, 1H, H_{Ar}), 7.31–7.53 (m, 5 H, H_{Ar}), 11.78 (s, 1H, NHC = O), 12.35 (br. s, 1H, CH_2COOH) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 33.61 (CH_2COOH), 34.40 (CH_2CH), 45.75 ($NHCH_2$), 103.15, 103.41, 103.64, 110.20, 110.34, 110.41, 110.44, 127.92, 128.63, 129.38, 132.78, 148.35, 150.91, 151.03, 151.35, 151.45, 151.41 (C_{Ar}), 173.18 (NHC = O), 176.66 (CH_2COOH) ppm. Calcd. for $C_{18}H_{16}F_2N_4O_2$, %: C 36.45; H 3.74; N 9.45; Found, %: C 36.34; H 3.70; N 9.39.

*4-[(2,4-Difluorophenyl)amino]-3-(4-phenyl-5-thioxo-4,5-dihydro-1 H-1,2,4-triazol-3-yl)butanoic acid (**16**)*
Compound **15** (0.4 g, 1 mmol) was dissolved in 4% NaOH solution (7 mL). Reaction was heated at reflux for 2 h, later sodium hydroxide was neutralized using glacier acetic acid until pH 6 was reached. Formed crystals were filtered, dried and recrystalized using 1,4-dioxane.

Brownish solid, yield 0.28 g, 71%, m. p. 142–143 °C. IR (KBr) ν 3387; 3138 (2x NH); 3094 (OH); 1696 (C = O); 1523 (C = N); 1269 (C = S) cm^{-1}. ^{1}H NMR (400 MHz, DMSO–d_6) δ 2.29–2.40, 2.67–2.77 (2 m, 2 H, CH_2COOH), 3.02–3.25, 3.54–3.94 (2 m, 3 H, CNCH overlaps with NHCH_2), 5.58 (s, 1H, CH_2N*H*), 6.02 (dd, *J* = 14.9, 9.3 Hz, 1H, H_{Ar}), 6.38–6.55 (m, 1H, H_{Ar}), 6.91–7.01 (m, 1H, H_{Ar}), 7.30–7.59 (m, 5 H, H_{Ar}), 11.65 (s, 1H, NHC = S), 12.27 (br. s, 1H, CH_2COO*H*) ppm. ^{13}C NMR (101 MHz, DMSO–d_6) δ 32.67 (*C*H_2COOH), 34.52 (CH_2CH), 46.35 (NHCH_2), 51.97, 103.19, 103.24, 110.15, 110.19, 110.36, 110.39, 110.48, 110.53, 110.56, 110.61, 128.56, 128.75, 128.77, 128.88, 129.08, 129.35, 132.64, 132.76, 133.98, 148.46, 148.58, 150.88, 150.98, 151.21, 151.32, 151.92, 155.17, 166.90, 168.32 (C_{Ar}); 174.78 (CH_2*C*OOH); 182.22 (C = S) ppm. Calcd. for $C_{18}H_{16}F_2N_4O_2S$, %: C 55.38; H 4.13; N 14.35; Found, %: C 55.29; H 4.09; N 14.29.

Biological activity

Cell culturing
The human triple-negative breast cancer MDA-MB-231 and human melanoma cell line A375 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human foreskin fibroblasts (HF) CRL-4001 were originally obtained from ATCC and kindly provided by Prof. Helder Santos (University of Helsinki, Finland). The primary prostate carcinoma PPC-1 cell line was kindly provided by Prof. Tambet Teesalu (University of Tartu, Estonia). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX (Gibco (Carlsbad, CA, USA)), supplemented with 10,000 U/mL penicillin, 10 mg/mL streptomycin (Gibco), and 10% fetal bovine serum (Gibco). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. They were used until the passage of 20.

Cytotoxicity
The compound effect on cancer cell viability was established using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co., St Louis, MO, USA) assay, as described elsewhere[35]. Briefly, the cells were seeded in triplicates in 96-well TC-treated flat bottom plates (MDA-MB-231, A375 and PPC-1: 4×10^3 cells/well; HF: 5×10^3 cells/well). The cells were treated with 100 µM of tested compounds or anticancer drugs sunitinib (≥95%, Santa Cruz Biotechnology, TX, USA), doxorubicin (> 97%, Merck, Austria) or cisplatin (Pt 64.5% min, Alfa Aesar, MA, USA) after 24 h of incubation. After 72 h, 20 µl of the MTT reagent was added to each well, and plates were incubated for an additional 3 hours in the incubator. Then, the formed formazan crystals were dissolved in DMSO (Sigma-Aldrich Co., St. Louis, MO, USA). The absorbance was determined with a multidetection microplate reader at 570 and 630 nm.

Using the same MTT procedure, the EC_{50} values of the most active hydrazones **7b**, **9a**, **9c**, **9e**, **9f** and **10** were established. The compound 2× serial dilutions, ranging from 50 µM to 1.56 µM, were added to the cells in triplicate (technical repetitions). The cells not treated with compounds were used as a negative control (their viability corresponded to 100%), and the positive control was the empty well without cells, representing the situation when all cells were killed (cell viability corresponded to 0%). After measuring the absorbance of the obtained MTT solutions, the half-maximal effective concentration (EC_{50}) values were calculated using the Hill equation. EC_{50} value was considered as the compound concentration, which resulted in a 50% reduction in the metabolic activity of cells. All experiments were repeated three times independently (biological repetitions).

Wound healing assay
'Wound healing' assay was used to evaluate the compound effect on selected cancer cell migration. In this assay, only the most active compounds **7b**, **9a**, **9c**, **9e**, and **10** were tested, and the procedure was performed as published elsewhere[38]. MDA-MB-231 cells were seeded at a density of 1×10^4 cells/well, and PPC-1 and A375 cells were seeded at a density of 2×10^4 cells/well in 24-well plates. After 2–3 days of incubation, once the cells reached 80–90% confluency, the scratch was made with a 100 µL pipette tip in the middle of each well. After washing the cells with PBS, the fresh medium containing 10 µM of compounds was added to the cells. As a negative control, a medium containing 0.1% DMSO was used. Photos of the 'wound' were made at the beginning of the experiment (0 h) and after the selected duration of incubation until the cells in control fully migrated into the 'wound area': 12, 24 and 36 h for MDA-MB-231 cells, 24 and 48 h for PPC-1 and A375 cells.

Effect in 3D cell cultures
The magnetic 3D Bioprinting method was used to form cancer cell spheroids as described elsewhere[39]. Briefly, the cells in a 6-well plate at 70% confluency were incubated with Nanoshuttle (n3D Biosciences, Inc., Houston, TX, USA) for 8–10 h. Then, the cells were trypsinized, centrifuged and seeded into the ultra-low attachment 96-well plate at a ratio of 1.5×10^3 cancer cells and 1.5×10^3 human fibroblasts/well. The plate was incubated for two days at 37 °C in a humidified atmosphere on a magnetic drive until the spheroids formed. Next, the medium with 10 µM of selected compounds **7b**, **9e**, **9f**, **10**, and doxorubicin hydrochloride (Abcam, Cambridge, UK) was added. Photos of spheroids were taken every two days up to 8 days using an Olympus IX73 inverted microscope (OLYMPUS CORPORATION, Tokyo, Japan), and analysis of spheroid size was performed using ImageJ, version 1.53o (National Institutes of Health, USA) and Microsoft Office Excel 2016 software (Microsoft Corporation, Redmond, WA, USA).

On the last day of the experiment, 20 µL of MTT reagent was added to each well. Following a 10-hour incubation, the medium was aspirated, and the formazan crystals formed were dissolved in 100 µL of DMSO overnight. The absorbance was determined with a multidetector microplate reader at 570 and 630 nm.

Statistical analysis

All biological experiments were repeated at least three times, calculating the mean and standard deviation. The data were processed using Microsoft Office Excel 2016 software (Microsoft Corporation, Redmond, WA, USA). Statistical analysis was performed by using Student's t-test. The level of significance was set as $p < 0.05$.

Data availability

Data is provided within the manuscript or supplementary information files.

Received: 11 February 2025; Accepted: 12 May 2025
Published online: 21 May 2025

References

1. Cancer Available online: https://www.who.int/news-room/fact-sheets/detail/cancer (accessed on 23 July 2024).
2. Ruf, S. et al. Novel β-Amino acid derivatives as inhibitors of cathepsin A. *J. Med. Chem.* **55**, 7636–7649. https://doi.org/10.1021/jm300663n (2012).
3. Rehman, M., Mahboob Ahmad, F. U. & Naveed Riaz, M. ??-Amino acids: role in human biology and medicinal Chemistry - A review. *Med. Chem.* **7** https://doi.org/10.4172/2161-0444.1000472 (2017).
4. Uesugi, S. et al. Pyrrocidine A, a metabolite of endophytic fungi, has a potent Apoptosis-Inducing activity against HL60 cells through caspase activation via the Michael addition. *J. Antibiot.* **69**, 133–140. https://doi.org/10.1038/ja.2015.103 (2016).
5. Jeličić, M. L., Kovačić, J., Cvetnić, M. & Mornar, A. Amidžić klarić, D. Antioxidant activity of pharmaceuticals: predictive QSAR modeling for potential therapeutic strategy. *Pharmaceuticals* **15**, 791. https://doi.org/10.3390/ph15070791 (2022).
6. Afzal, O. et al. Analgesic and anticancer activity of Benzoxazole clubbed 2-Pyrrolidinones as novel inhibitors of monoacylglycerol lipase. *Molecules* **26**, 2389. https://doi.org/10.3390/molecules26082389 (2021).
7. Muneer, S., Memon, S., Pahnwar, Q. K., Bhatti, A. A. & Khokhar, T. S. Synthesis and investigation of antimicrobial properties of pyrrolidine appended Calix[4]Arene. *J. Anal. Sci. Technol.* **8** (3). https://doi.org/10.1186/s40543-017-0111-3 (2017).
8. Khalifa, M. E. et al. Synthesis, antibacterial, and anti HepG2 cell line human hepatocyte carcinoma activity of some new potentially Benzimidazole-5-(Aryldiazenyl)Thiazole derivatives. *Molecules* **23** (3285). https://doi.org/10.3390/molecules23123285 (2018).
9. Rollas, S. & Küçükgüzel, S. G. Biological activities of hydrazone derivatives. *Molecules* **12**, 1910–1939. https://doi.org/10.3390/12081910 (2007).
10. Işik, A. et al. Benzimidazole-Hydrazone derivatives: synthesis, in vitro anticancer, antimicrobial, antioxidant activities, in Silico DFT and ADMET studies. *J. Mol. Struct.* **1270** https://doi.org/10.1016/j.molstruc.2022.133946 (2022).
11. Xing, A. et al. Anticancer and antioxidant activities of novel heterocyclic phenolic hydrazone based derivatives: investigation of DFT calculation, molecular Docking and Drug-Likeness studies. *J. Mol. Struct.* **1319**, 139523. https://doi.org/10.1016/j.molstruc.2024.139523 (2025).
12. Chandra, G., Singh, D. V., Mahato, G. K. & Patel, S. Fluorine-a small magic bullet atom in the drug development: perspective to FDA approved and COVID-19 recommended drugs. *Chem. Pap.* **77**, 4085–4106. https://doi.org/10.1007/s11696-023-02804-5 (2023).
13. Liu, H. N. et al. Synthetic approaches and application of representative clinically approved Fluorine-Enriched Anti-Cancer medications. *Eur. J. Med. Chem.* **276**, 116722. https://doi.org/10.1016/j.ejmech.2024.116722 (2024).
14. Afifi, N. & Barrero, C. A. Understanding breast Cancer aggressiveness and its implications in diagnosis and treatment. *JCM* **12** (1375). https://doi.org/10.3390/jcm12041375 (2023).
15. Bray, F. et al. Global Cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **68**, 394–424. https://doi.org/10.3322/caac.21492 (2018).
16. Brothman, A. R., Wilkins, P. C., Sales, E. W. & Somers, K. D. Metastatic properties of the human prostatic cell line, PPC-1, in athymic nude mice. *J. Urol.* **145**, 1088–1091. https://doi.org/10.1016/S0022-5347(17)38540-3 (1991).
17. International Agency for Research on Cancer. Cancer Tomorrow Estimated Number of New Cases from 2020 to 2040 of Melanoma of Skin and Non-Melanoma Skin Cancer. Available online: https://gco.iarc.who.int/today/ (accessed on 25 July 2024).
18. Lopes, J., Rodrigues, C. M. P., Gaspar, M. M. & Reis, C. P. Melanoma management: from epidemiology to treatment and latest advances. *Cancers* **14**, 4652. https://doi.org/10.3390/cancers14194652 (2022).
19. Treating Melanoma Skin Cancer | How Is Melanoma Treated?. Available online: July (2024). https://www.cancer.org/cancer/types/melanoma-skin-cancer/treating.html (accessed on 26.
20. Bouchereau, S. et al. Impact of prior treatment with immune checkpoint inhibitors on Dacarbazine efficacy in metastatic melanoma. *Br. J. Cancer.* **125**, 948–954. https://doi.org/10.1038/s41416-021-01486-8 (2021).
21. Rossi, S. et al. TNF-Alpha and metalloproteases as key players in melanoma cells aggressiveness. *J. Exp. Clin. Cancer Res.* **37**, 326. https://doi.org/10.1186/s13046-018-0982-1 (2018).
22. Ghasemi, M., Turnbull, T., Sebastian, S. & Kempson, I. The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and Single-Cell analysis. *IJMS* **22** (12827). https://doi.org/10.3390/ijms222312827 (2021).
23. Balandis, B. et al. Synthesis and Structure–Affinity relationship of chlorinated Pyrrolidinone-Bearing benzenesulfonamides as human carbonic anhydrase inhibitors. *Bioorg. Chem.* **97**, 103658. https://doi.org/10.1016/j.bioorg.2020.103658 (2020).
24. SUNITINIB. A Comprehensive Guide for Patients Available online: April (2025). https://clinicaltrials.eu/inn/sunitinib/ (accessed on 22.
25. Brown, A., Kumar, S. & Tchounwou, P. B. Cisplatin-Based chemotherapy of human cancers. *J. Cancer Sci. Ther.* **11**, 97 (2019).
26. Kciuk, M. et al. Doxorubicin—An agent with multiple mechanisms of anticancer activity. *Cells* **12**, 659. https://doi.org/10.3390/cells12040659 (2023).
27. Bedia, C., Casas, J., Andrieu-Abadie, N., Fabriàs, G. & Levade, T. Acid ceramidase expression modulates the sensitivity of A375 melanoma cells to Dacarbazine. *J. Biol. Chem.* **286**, 28200–28209. https://doi.org/10.1074/jbc.M110.216382 (2011).
28. Piskorz, W. M., Krętowski, R., Cechowska-Pasko, M. & Marizomib Salinosporamide A) promotes apoptosis in A375 and G361 melanoma Cancer cells. *Mar. Drugs.* **22**, 315. https://doi.org/10.3390/md22070315 (2024).
29. Cordaro, F. G. et al. Phenotype characterization of human melanoma cells resistant to Dabrafenib. *Oncol. Rep.* **38**, 2741–2751. https://doi.org/10.3892/or.2017.5963 (2017).
30. Januškevičienė, I. & Petrikaitė, V. Heterogeneity of breast cancer: the importance of interaction between different tumor cell populations. *Life Sci.* **239**, 117009. https://doi.org/10.1016/j.lfs.2019.117009 (2019).
31. Januškevičienė, I. & Petrikaitė, V. Interaction of phenotypic sublines isolated from Triple-Negative breast Cancer cell line MDA-MB-231 modulates their sensitivity to Paclitaxel and doxorubicin in 2D and 3D assays. *Am. J. Cancer Res.* **13**, 3368–3383 (2023).
32. Bashi, M., Madanchi, H. & Yousefi, B. Investigation of cytotoxic effect and action mechanism of a synthetic peptide derivative of rabbit Cathelicidin against MDA-MB-231 breast Cancer cell line. *Sci. Rep.* **14**, 13497. https://doi.org/10.1038/s41598-024-64400-1 (2024).
33. Riahi, R., Yang, Y., Zhang, D. D. & Wong, P. K. Advances in Wound-Healing assays for probing collective cell migration. *SLAS Technol.* **17**, 59–65. https://doi.org/10.1177/2211068211426550 (2012).

34. Daunys, S., Matulis, D. & Petrikaitė, V. Synergistic activity of Hsp90 inhibitors and anticancer agents in pancreatic Cancer cell cultures. *Sci. Rep.* **9**, 16177. https://doi.org/10.1038/s41598-019-52652-1 (2019).
35. Bytautaite, M. & Petrikaite, V. Comparative study of lipophilic Statin activity in 2D and 3D in vitro models of human breast Cancer cell lines MDA-MB-231 and MCF-7. *Onco Targets Ther.* **13**, 13201–13209. https://doi.org/10.2147/OTT.S283033 (2020).
36. Li, L. & Lu, Y. Optimizing a 3D culture system to study the interaction between epithelial breast Cancer and its surrounding fibroblasts. *J. Cancer*. **2**, 458–466. https://doi.org/10.7150/jca.2.458 (2011).
37. Mittler, F. et al. High-Content monitoring of drug effects in a 3D spheroid model. *Front. Oncol.* **7**, 293. https://doi.org/10.3389/fonc.2017.00293 (2017).
38. Skaraitė, I., Maccioni, E. & Petrikaitė, V. Anticancer activity of Sunitinib analogues in human pancreatic Cancer cell cultures under normoxia and hypoxia. *IJMS* **24** (5422). https://doi.org/10.3390/ijms24065422 (2023).
39. Šedbarė, R., Janulis, V., Pavilonis, A. & Petrikaite, V. Comparative in vitro study: assessing phytochemical, antioxidant, antimicrobial, and anticancer properties of Vaccinium Macrocarpon Aiton and Vaccinium Oxycoccos L. Fruit extracts. *Pharmaceutics* **16**, 735. https://doi.org/10.3390/pharmaceutics16060735 (2024).

Author contributions

Author Contributions: Conceptualization, V.M. and V.P.; methodology, V.M., and V.P.; formal analysis, G.P., B.G., U.E., and V.P.; investigation, G.P., B.G., U.E., and V.P.; resources, V.M. and V.P.; writing—original draft preparation, G.P., B.G., and V.P.; writing—review and editing, B.G., and V.P.; visualization, B.G. and V.P.; supervision, V.M. and V.P.; funding acquisition, V.M. and V.P. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-02106-8.

Correspondence and requests for materials should be addressed to V.M. or V.P.