*https://doi.org/10.31925/farmacia.2022.2.10* **ORIGINAL ARTICLE**

# CYTOTOXIC EVALUATION OF ORIGINAL PYRAZOLO[3,4-*d*]THIAZOLES AND PYRAZOLO[3,4-*c*]PYRAZOLES

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## Abstract

A concise and versatile synthetic strategy for the synthesis of original substituted heterobicycles was developed, relying on the use of aromatic hydrazines in one-pot condensations and Ullmann-type intramolecular cyclization sequences, as well as on chemo-selective brominations and palladium-mediated cross-coupling reactions. In this study, the two series of novel heterobicycles were tested for their cytotoxic potential. The compounds were structurally characterized by nuclear magnetic resonance spectrometry ($^1$H NMR and $^{13}$C NMR), infrared spectroscopy (IR) and high-resolution mass spectrometry (HRMS). The $IC_{50}$ values of these compounds on five tumour cell lines (A-549, HS-683, MCF-7, SK-MEL-28 and B16-F1) were obtained by MTT colorimetric assay. Six of the compounds exhibited excellent activity compared to 5-fluorouracil against two of the five cell lines tested, with $IC_{50}$ values ranging from 0.1 to 10 μM.

## Rezumat

A fost dezvoltată o strategie concisă și versatilă pentru sinteza de heterociclii substituiți. Aceasta se bazează pe utilizarea de hidrazine aromatice monosubstituite folosite în reacții *one-pot* de condensare și de ciclizare intramoleculară de tip Ullmann, precum și pe secvențe de bromurare chemoselectivă și reacții de cuplare încrucișată mediată de paladiu. În acest studiu, cele două serii de heterociclii au fost testate pentru potențialul lor citotoxic. Compușii au fost caracterizați structural prin spectrometrie de rezonanță magnetică nucleară ($^1$H RMN și $^{13}$C RMN), spectroscopie în infraroșu (IR) și spectrometrie de masă de înaltă rezoluție (HRMS). Valorile $IC_{50}$ ale acestor compuși pentru cele cinci linii de celule tumorale luate în considerare (A-549, HS-683, MCF-7, SK-MEL-28 și B16-F1) au fost obținute prin testul colorimetric MTT. Șase dintre compuși au prezentat o activitate mai bună comparativ cu standardul 5-fluorouracil pentru două dintre cele cinci linii celulare testate, cu valori $IC_{50}$ cuprinse între 0,1 și 10 μM.

**Keywords**: pyrazole, thiazole, hydrazine, one-pot, cytotoxic, cancer

## Introduction

Pyrazole and thiazole scaffolds are known to possess a central interest in the therapeutic area namely for their biological properties, including anti-tumour activity [1, 2]. The strong pharmacophore aspect of these simple heterocycles is underlined by several marketed anti-inflammatory drugs such as celecoxib and fanetizole (Figure 1) [3]. In the scope of combining the potential of the two molecules, we focused on the synthesis of original cytotoxic endowed structures, highlighting poorly described fused [5:5] ring systems: pyrazolo-[3,4-*d*]thiazoles and pyrazolo[3,4-*c*]pyrazoles [4].

The access to the aforementioned heterobicycles is described by various methods [5-8] possessing several downsides including long reaction times, complex starting materials, modest yields or long synthetic pathways. Moreover, few examples were studied from a therapeutic standpoint [9-12], some of which had limited [9, 12], or no biological (antiviral, anti-tumoural) activity [11] (Figure 1).

In this context, starting from inexpensive starting materials, two efficient strategies for the synthesis of novel pyrazolo[3,4-*d*]thiazoles and pyrazolo[3,4-*c*]-pyrazoles were developed. The two approaches rely on first the use of aromatic hydrazines in a one-pot condensation and an Ullmann-type intramolecular cyclization sequence, followed by a palladium-mediated cross-coupling reactions for further modulations (Figure 2).

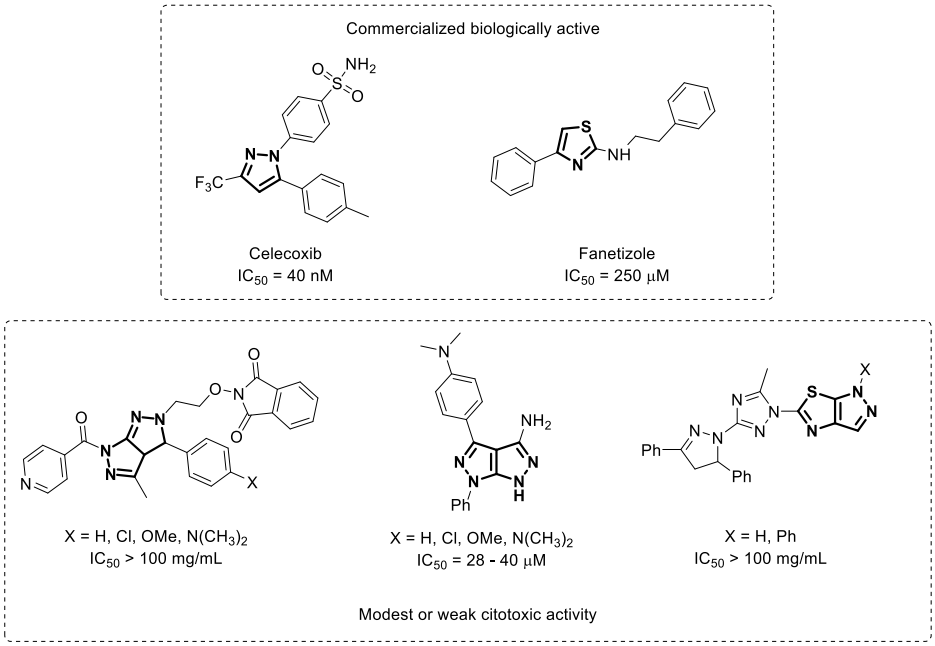



**Figure 1.**
Representative examples of pyrazole and thiazole derivatives and their biological potential

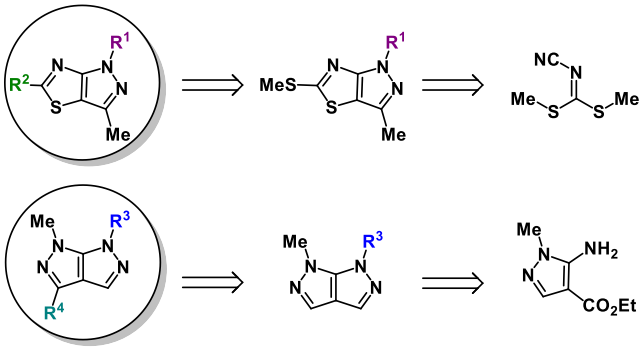


**Figure 2.**
Retrosynthetic approaches

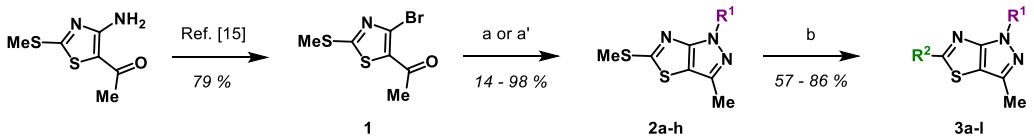
## Materials and Methods

*Chemistry*

All reagents and organic solvents were purchased from commercial suppliers (Fluorochem – UK, Sigma Aldrich – France) and were used without further purification. Microwave assisted reactions were carried out in a Biotage Initiator microwave synthesis instrument (Biotage – USA) and temperatures were measured by an IR sensor. Solvents mentioned as dry were purified with a dry station GT S100 (Glass Technology – Switzerland) immediately prior to use. The reactions were monitored by thin-layer chromatography (TLC) analysis using 0.2 mm precoated Kieselgel 60 F254 (Merck – USA) silica gel plates visualized with a UV254 lamp. Column chromatography was performed on silica gel 60 (230 - 400 mesh, 40 - 63 μm). Solvent ratios for chromatography purification are reported as v/v ratios and are indicated for each compound. Melting points (mp [°C]) were taken on samples in open capillary tubes and are uncorrected on an IA9200 Thermo Scientific Electrothermal Melting Point (Thermo Scientific – USA) apparatus/instrument. Infrared analyses were determined on a Thermo Scientific ATR Nicolet iS10 (Thermo Fischer – USA) and interpreted using OMNIC software. $^{1}$H NMR and $^{13}$C NMR spectra were recorded either on a Bruker ULTRASHIELD® Plus 400 MHz (Bruker – Germany) spectrometer ($^{13}$C, 100 MHz) or on a Bruker AVANCE 250 MHz (Bruker – Germany) spectrometer ($^{13}$C, 62.9 MHz), as solutions in deuterated solvents. Unless otherwise indicated, chemical shifts ($\delta$) are reported in parts *per* million (ppm) values, and coupling constants ($J$) are reported in Hertz. Peak multiplicities are designated by the following abbreviations: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; dd = double doublet and br = broadened. High-resolution mass spectrometry analyses (HRMS) were performed on a Maxis Bruker 4G Spectrometer (Bruker, Germany). CLogP values were established using MolDesc software, http://moldesc.icoa.fr/ (France).

*Synthesis of pyrazolo[3,4-d]thiazoles*

Starting from dimethyl-l-*N*-cyanodithioiminocarbonate and relying on a three step one-pot sequence [13, 14], the amino thiazole scaffold was obtained with excellent yield. A Sandmeyer reaction [15] of the latter gave access to the brominated corresponding key intermediate **1**. Condensation reactions with mono-substituted aromatic hydrazines followed by an intra-molecular copper-catalyzed, ligand-free N-cyclizations allowed the formation of original N1 modulated pyrazolo[3,4-*d*]thiazoles **2a-h**. The fused system was submitted to a Liebeskind-Srogl cross-coupling reaction, by using various monosubstituted aromatic boronic acids, under microwave irradiation giving the tri-functionalized structures **3a-l** (Figure 3) [16].




**2a**: $R^1$ = Ph
**2b**: $R^1$ = 4-$NO_2$-$C_6H_4$
**2c**: $R^1$ = 4-$CH_3$-$C_6H_4$
**2d**: $R^1$ = 4-$CH_3O$-$C_6H_4$
**2e**: $R^1$ = 4-F-$C_6H_4$
**2f**: $R^1$ = 3-F-$C_6H_4$
**2g**: $R^1$ = 2-F-$C_6H_4$
**2h**: $R^1$ = 4-$CF_3$-$C_6H_4$

**3a**: $R^1$ = Ph, $R^2$ = 4-$CH_3O$-$C_6H_4$
**3b**: $R^1$ = Ph, $R^2$ = 3-$CH_3O$-$C_6H_4$
**3c**: $R^1$ = Ph, $R^2$ = 2-$CH_3O$-$C_6H_4$
**3d**: $R^1$ = Ph, $R^2$ = 4-$CH_3$-$C_6H_4$
**3e**: $R^1$ = Ph, $R^2$ = Ph
**3f**: $R^1$ = Ph, $R^2$ = 4-CN-$C_6H_4$
**3g**: $R^1$ = Ph, $R^2$ = 4-$CF_3$-$C_6H_4$
**3h**: $R^1$ = 4-$CH_3$-$C_6H_4$, $R^2$ = 4-$CH_3O$-$C_6H_4$
**3i**: $R^1$ = 4-$CH_3O$-$C_6H_4$, $R^2$ = 4-$CH_3O$-$C_6H_4$
**3j**: $R^1$ = 4-$CH_3O$-$C_6H_4$, $R^2$ = 4-$CF_3$-$C_6H_4$
**3k**: $R^1$ = 4-F-$C_6H_4$, $R^2$ = 4-$CH_3O$-$C_6H_4$
**3l**: $R^1$ = 4-$CF_3$-$C_6H_4$, $R^2$ = 4-$CH_3O$-$C_6H_4$

**Figure 3.**
Synthesis of modulated pyrazolo[3,4-*d*]thiazoles
Reagents and conditions: a) salt free hydrazine, AcOH, EtOH, reflux, 18 h and then CuI, $K_3PO_4$, DMA, 100°C, 2 h; a') hydrazine hydrochloride salt, NaOAc, EtOH, reflux, 18 h and then CuI, $K_3PO_4$, DMA, 100°C, 2 h; b) $R^2B(OH)_2$, $Pd(PPh_3)_4$, CuTC, DMF, 140°C, MW, 2 h

From the diverse array of the newly formed pyrazolo-[3,4-*d*]thiazoles, the removal of N1 *p*-methoxyphenyl ring of **2d** and **3a** were tried to obtain the corresponding original pyrazolo[3,4-*d*]thiazoles **4a** and **4b** (Figure 4).

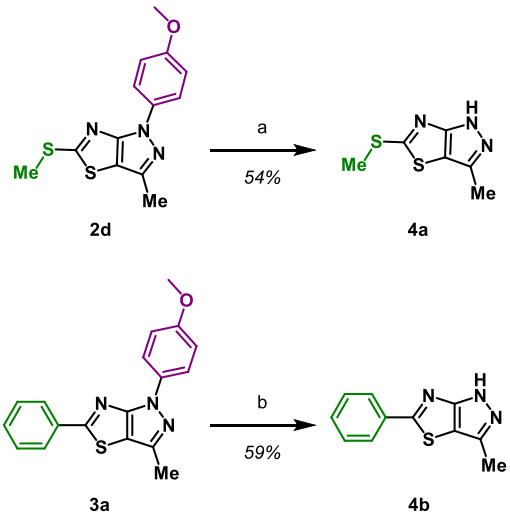
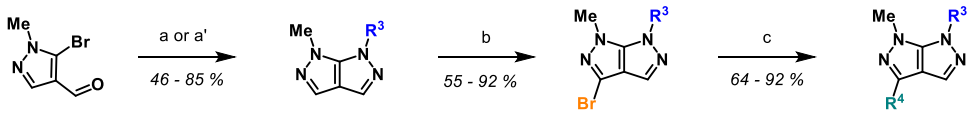



**Figure 4.**
Synthesis of N1 deprotected pyrazolo[3,4-*d*]-thiazoles
Reagents and conditions: a) CAN, MeCN/$H_2O$ (2:1), 0°C, 1 h; b) CAN, MeCN/$H_2O$ (2:1), rt, 3 h

After 1 hour at 0°C in presence of ceric ammonium nitrate (CAN) [17], **2d** was completely converted into **4a** in a moderate yield. Concerning **3a**, low temperatures seemed to render the reaction sluggish and only after 3 hours at room temperature a total conversion of the starting material was reached, leading to **4b** in a good yield.

*Synthesis of pyrazolo[3,4-c]pyrazoles*

The robust synthetic approach starts from commercially available ethyl 5-amino-1-methyl-1*H*-pyrazole-4-carboxylate which after a Sandmeyer reaction is converted to its brominated analogue [18]. DIBAL reduction of the ester group to the corresponding allylic alcohol, followed by its mild $MnO_2$ oxidation furnished the synthon **5** without any purification step [19]. The latter pyrazole framework was involved in a condensation transformation with various mono-substituted hydrazines, followed by a ligand-free Ullmann-type cyclization reaction under microwave irradiation, in a one-pot manner. Original pyrazolo-[3,4-*c*]pyrazoles **6a-i** were thus obtained and further functionalized by selective bromination in position 3 (Figure 5) [20]. Once the bromine atom inserted, a Suzuki-Miyaura cross-coupling reaction was performed [21], allowing the insertion of several (hetero)aryl motifs with good to excellent yield (Figure 5).




6a: $R^3$ = Ph
6b: $R^3$ = 4-$CH_3$-$C_6H_4$
6c: $R^3$ = 4-$CH_3O$-$C_6H_4$
6d: $R^3$ = 4-CN-$C_6H_4$
6e: $R^3$ = 4-$CF_3$-$C_6H_4$
6f: $R^3$ = 4-F-$C_6H_4$
6g: $R^3$ = 3-F-$C_6H_4$
6h: $R^3$ = 2-F-$C_6H_4$
6i: $R^3$ = 4-Py

7a: $R^3$ = Ph
7b: $R^3$ = 4-$CH_3O$-$C_6H_4$
7c: $R^3$ = 4-$CF_3$-$C_6H_4$

8a: $R^3$ = Ph, $R^4$ = 4-$CH_3$-$C_6H_4$
8b: $R^3$ = Ph, $R^4$ = 3-$CH_3$-$C_6H_4$
8c: $R^3$ = Ph, $R^4$ = 2-$CH_3$-$C_6H_4$
8d: $R^3$ = Ph, $R^4$ = Ph
8e: $R^3$ = Ph, $R^4$ = 4-$CH_3O$-$C_6H_4$
8f: $R^3$ = Ph, $R^4$ = 4-$CF_3$-$C_6H_4$
8g: $R^3$ = Ph, $R^4$ = 4-CN-$C_6H_4$
8h: $R^3$ = Ph, $R^4$ = 3-F-$C_6H_4$
8i: $R^3$ = Ph, $R^4$ = 3-Tiophenyl
8j: $R^3$ = Ph, $R^4$ = 4-Py
8k: $R^3$ = Ph, $R^4$ = Styryl
8l: $R^3$ = 4-$CH_3O$-$C_6H_4$, $R^4$ = 4-CN-$C_6H_4$
8m: $R^3$ = 4-$CH_3O$-$C_6H_4$, $R^4$ = 4-Py
8n: $R^3$ = 4-$CH_3O$-$C_6H_4$, $R^4$ = 4-$CH_3O$-$C_6H_4$
8o: $R^3$ = 4-$CF_3$-$C_6H_4$, $R^4$ = 4-CN-$C_6H_4$
8p: $R^3$ = 4-$CF_3$-$C_6H_4$, $R^4$ = 4-Py
8q: $R^3$ = 4-$CF_3$-$C_6H_4$, $R^4$ = 4-$CH_3O$-$C_6H_4$
8r: $R^3$ = 4-$CF_3$-$C_6H_4$, $R^4$ = 4-$CH_3$-$C_6H_4$

**Figure 5.**
Synthesis of modulated pyrazolo[3,4-*c*]pyrazoles
Reagents and conditions: a) salt free hydrazine, AcOH, EtOH, reflux, 18 h and then CuI, $K_2CO_3$, DMF, 150°C, 1 h; a') hydrazine hydrochloride salt, NaOAc, EtOH, reflux, 18 h and then CuI, $K_2CO_3$, DMF, 150°C, 1 h; b) NBS, MeCN, reflux, MW or conventional heating, 2 - 4 h; c) $R^4B(OH)_2$, $Pd(PPh_3)_4$, $Cs_2CO_3$, 1,4-dioxane/EtOH/$H_2O$ (3:1:0.5), MW, 140°C, 1 - 2 h

*Biological evaluation*

With regards to the undermined therapeutic potential of these frameworks, 52 heterobicycles were synthesized.

**Table I**

Calculated LogP values for the synthesized compounds

| Compound | CLogP (MolDesc) | Compound | CLogP (MolDesc) |
|---|---|---|---|
| **2a** | 3.5 | **3k** | 4.6 |
| **2b** | 3.4 | **3l** | 4.4 |
| **2c** | 3.8 | **4a** | 2.0 |
| **2d** | 3.5 | **4b** | 3.0 |
| **2e** | 3.7 | **6a** | 1.8 |
| **2f** | 3.7 | **6b** | 2.1 |
| **2g** | 3.7 | **6c** | 1.8 |
| **2h** | 4.5 | **6d** | 1.6 |
| **3a** | 4.5 | **6e** | 2.8 |
| **3b** | 4.5 | **6f** | 1.9 |
| **3c** | 4.5 | **6g** | 1.9 |
| **3d** | 4.8 | **6h** | 1.9 |
| **3e** | 4.5 | **6i** | 1.2 |
| **3f** | 4.3 | **7a** | 2.5 |
| **3g** | 5.5 | **7b** | 2.5 |
| **3h** | 4.8 | **7c** | 3.5 |
| **3i** | 4.5 | **8a** | 3.7 |
| **3j** | 5.8 | **8b** | 3.7 |
| **8c** | 3.7 | **8k** | 3.9 |
| **8d** | 3.4 | **8l** | 3.3 |
| **8e** | 3.4 | **8m** | 2.8 |
| **8f** | 4.4 | **8n** | 3.4 |
| **8g** | 3.3 | **8o** | 4.3 |
| **8h** | 3.6 | **8p** | 3.8 |
| **8i** | 3.5 | **8q** | 4.5 |
| **8j** | 2.8 | **8r** | 4.8 |

The lipophilicity assessment of the synthesized compounds was performed, as this parameter is crucially linked to ADME properties. Relying thus on MolDesc software, the membrane-water partition coefficient (LogP) was calculated (Table I).

Given the obtained CLogP results, these heterobicycles seem to be suitable for future development of drug-like candidates [22]. Starting from this panel, 46 promising compounds were chosen for *in vitro* testing for their potential cytotoxic activity using five cancer cell lines by MTT (thiazolyl blue tetrazolium bromide) assay.

## Results and Discussion

*Chemistry*

Compounds **1** - **3a-l** and **5** - **8a-r** were obtained following the literature [16, 23].

*3-Methyl-5-(methylthio)-1H-pyrazolo[3,4-d]thiazole (**4a**)*

To a stirred solution of 1-(4-methoxyphenyl)-3-methyl-5-(methylthio)-1*H*-pyrazolo[3,4-*d*]thiazole **2d** (100 mg, 0.34 mmoles) in acetonitrile (5 mL), an aqueous solution (2.5 mL) of ceric ammonium (IV) nitrate (565 mg, 1.03 mmoles, 3.0 eq.) was added. The reaction mixture was cooled to 0°C and left under stirring for one hour. Extraction with DCM was followed by drying of the collected organic layers on $MgSO_4$. The obtained crude mixture was purified by silica gel column chromatography using appropriate solvents to give the title compound (35 mg, 54%) as a light brown solid. $R_f$: 0.3 (5/5 PE/EtOAc). M.p.: 171 - 172ºC. Column chromatography eluents: PE/EtOAc = 5/5.

$^1$H NMR (250 MHz, Chloroform-*d*): $\delta$ 12.15 (br s, 1H), 2.77 (s, 3H), 2.49 (s, 3H). $^{13}$C NMR (101 MHz, Chloroform-*d*): $\delta$ 172.5 (C), 109.6 (C), 15.0 (S-$CH_3$), 10.9 ($CH_3$). Note: 2 quaternary carbon atoms were not observed despite longer experiences, probably due to the hindrance of the nearby heteroatoms. IR *v* ($cm^{-1}$): 3176, 3107, 2919, 2858, 1586, 1438, 1397, 1287, 1072, 957, 805. HRMS (ESI): (*m/z*) $[M+H]^+$ calculated for $[C_6H_8N_3S_2]^+$ 186.0154; found 186.0154.

*3-Methyl-5-phenyl-1H-pyrazolo[3,4-d]thiazole (**4b**)*

To a stirred solution of 1-(4-methoxyphenyl)-3-methyl-5-phenyl-1*H*-pyrazolo[3,4-*d*]thiazole **3a** (1.11 g, 3.46 mmoles) in acetonitrile (54 mL) an aqueous solution (27 mL) of ceric ammonium (IV) nitrate (7.6 g, 13.85 mmoles, 4.0 eq.) was added. The reaction mixture was left under stirring for 3 hours. Extraction with DCM was followed by drying of the collected organic layers on $MgSO_4$. The obtained crude mixture was purified by silica gel column chromatography using appropriate solvents to give the title compound (440 mg, 59%) as a light brown solid. $R_f$: 0.2 (8/2 PE/EtOAc). M.p.: 172 - 173ºC. Column chromatography eluents: PE/ EtOAc = 5/5. $^1$H NMR (400 MHz, Chloroform-*d*): $\delta$ 11.27 (br s, 1H), 8.07 - 7.96 (d, *J* = 8.0 Hz, 2H), 7.53 - 7.42 (overlapped peaks, t and t, 3H), 2.59 (s, 3H, $H_9$). $^{13}$C NMR (101 MHz, Chloroform-*d*): $\delta$ 174.5 (C), 134.2 (C), 131.1 ($C_{Ph}$), 129.1 (2 x $C_{Ph}$), 127.1 (2 x $C_{Ph}$), 111.6 (C), 13.0 ($CH_3$). Note: 2 quaternary carbon atoms were not observed despite longer experiences, probably due to the hindrance of the nearby heteroatoms. IR *v* ($cm^{-1}$): 3196, 3111, 2849, 2775, 1601, 1451, 1299, 1243, 1176, 1087, 944, 816, 759. HRMS (ESI): (*m/z*) $[M+H]^+$ calculated for $[C_{11}H_{10}N_3S]^+$ 216.0589; found 216.0588.

*Biological evaluation*

From the plethora of pyrazolo[3,4-*d*]thiazoles and pyrazolo[3,4-*c*]pyrazoles derivatives, 46 compounds were evaluated *in vitro* against several cancerous cellular lines: A549 - lung carcinoma, HS-683 – glioma cancer, MCF-7 - breast carcinoma, SK-MEL-28 and B16-F1 - melanoma cancer (Table II).

Among this panel, compounds **2b**, **3h**, **3k**, **3l**, **8d**, **8g**, **8h**, **8l**, **8n**, **8q** and **8r** proved strong inhibitory activities against the tested cellular lines, with $IC_{50}$ values between 0.1 - 1 μM and 1 - 10 μM ($IC_{50}$ defined as the concentration inducing a 50% decrease of cell growth after 3 days of incubation). Moreover, hetero-bicycles **2b**, **3l**, **8g**, **8l**, **8n** and **8q** exhibited higher anti-tumour activity than the positive control 5-fluoro-uracil against HS-683 and SK-MEL-28 cells with $IC_{50}$ values between 0.1 μM and 10 μM. These compounds bear strong electron-withdrawing groups such as $NO_2$, $CF_3$ or electron-donating groups such as $CH_3O$ as commune features. Although their presence appears to be crucial for the activity, it is not the only structural determinant. In addition, compound **2b** was the only active 3-methyl-5-(methylthio)-pyrazolo[3,4-*d*]thiazole tested against the HS-683 cellular line ($IC_{50}$ values between 0.5 and 1 μM) whilst compound **3l** was the most effective framework concerning the same cellular line ($IC_{50}$ values between 0.1 and 0.5 μM). The latter exhibited also strong cytotoxicity for A549 as well as noticeable action for the MCF-7 cellular lines. Limited activity of compound **2h** was noticed for the same cellular line ($IC_{50}$ values between 50 and 100 μM).

Insertion of mono-substituted aromatic moieties onto pyrazolo[3,4-*d*]thiazole or deprotection reactions of these scaffolds lead to compounds which possessed only moderate activities ($IC_{50}$ between 10 and 50 or 50 and 100 μM) (**3a**, **3b**, **3c**, **3f**, **3h**, **3k** and **4b**) for the MCF-7 cellular line.

Tested bipyrazoles **6a**, **6b**, **6e** and **6i** showed no anti-tumour effect. Chemoselective bromination leads only to modest activities for compounds **7b** and **7c** ($IC_{50}$ = 50 - 100 μM). However, after the aromatic coupling reaction, several compounds proved to be active against one (**8d** and **8h** for A-594 cell line) or several cell lines (**8g**, **8l**, **8n**, **8q** and **8r**).

The presence of the methyl group – on the aromatic ring found on position 3 – in the *para* (**8a**) and *meta* (**8b**) sites gave activity signs solely for the A-549 cell line. Interestingly, the *ortho*-methyl analogue (**8c**) elicited extended efficacy also towards HS-683 and MCF-7 cell lines. To be noticed that replacing methyl group from the *meta* position on the phenyl ring by the fluorine atom (**8h**) is consistent with an increased anti-tumour effect for the A-549 cell line.

No difference in terms of cytotoxic response was noticed when either $CH_3O$- (**8e**) or $CF_3$- (**8f**) groups were used on the position 3 phenyl nucleus. However, the insertion of the CN- substituent (**8g**) was associated with noticeable results for three cellular lines (A-549, HS-683 and MCF-7). When the phenyl substituent inserted on the position 3 of the bipyrazole is deprived of functional groups (**8d**), a good response was seen for A-549 cell line and respectively a moderate one for HS-683, MCF-7 and B16-F1 cell lines.

The presence of the 3-thiophenyl motif (**8i**) or the styryl moiety (**8k**) instead of the phenyl ring seems to evoke a similar anti-tumour response of 10 or 50 up to 100 μM (MCF-7 and B16-F1 cell lines). Conversely, grafting the 4-pyridine heterocycle (**8j**) shifts the modest activity selectively towards the A-549 cell line.

Using the functional groups tandem 4-$CH_3O$- (on the phenyl scaffold found on position 6) and 4-CN- (on the phenyl moiety found on position 3) was linked to interesting biological responses for most of the tested cellular lines (**8l:** $IC_{50}$ = 5 - 10 μM). Replacing either of the aforementioned substituents with electron-donating groups (**8q** and **8r**), electron-withdrawing ones (**8o**) or even heterocycles (**8m** and **8p**) lead to a decrease of the cytotoxic effect, with one exception. Substituting 4-CN- group by 4-$CH_3$-O- (**8n**) leads to similar activities as compound **8l**. However, the response

concerning HS-683 line is completely lost whilst noticeable effect was noticed for the SK-MEL-28 cellular line.

**Table II**

Cytotoxic activity of the synthesized compounds expressed as $IC_{50}$ (μM)

| Compound | Cancer cell lines $IC_{50}$ (μM)[a] | | | | |
|---|---|---|---|---|---|
| | A-549[b] | HS-683[c] | MCF-7[d] | SK-MEL-28[e] | B16-F1[f] |
| **2a** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **2b** | > 100 | 0.5 - 1 | > 100 | > 100 | > 100 |
| **2c** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **2d** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **2e** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **2f** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **2g** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **2h** | > 100 | > 100 | 50 - 100 | > 100 | > 100 |
| **3a** | > 100 | > 100 | 50 - 100 | > 100 | > 100 |
| **3b** | > 100 | > 100 | 10 - 50 | > 100 | > 100 |
| **3c** | > 100 | 10 - 50 | 50 - 100 | > 100 | > 100 |
| **3d** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **3f** | 50 - 100 | > 100 | 50 - 100 | > 100 | > 100 |
| **3g** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **3h** | 5 - 10 | 5 - 10 | 10 - 50 | > 100 | > 100 |
| **3i** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **3j** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **3k** | 5 - 10 | 10 - 50 | 50 - 100 | 50 - 100 | > 100 |
| **3l** | 5 - 10 | 0.1 - 0.5 | 10 - 50 | > 100 | > 100 |
| **4a** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **4b** | 50 - 100 | > 100 | 50 - 100 | > 100 | > 100 |
| **6a** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **6b** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **6e** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **6i** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **7a** | > 100 | > 100 | > 100 | > 100 | > 100 |
| **7b** | 50 - 100 | > 100 | > 100 | > 100 | > 100 |
| **7c** | 50 - 100 | > 100 | > 100 | > 100 | > 100 |
| **8a** | 50 - 100 | > 100 | > 100 | > 100 | > 100 |
| **8b** | 50 - 100 | > 100 | > 100 | > 100 | > 100 |
| **8c** | 10 - 50 | 50 - 100 | 50 - 100 | > 100 | > 100 |
| **8d** | 1 - 5 | 50 - 100 | 10 - 50 | > 100 | 50 - 100 |
| **8e** | 10 - 50 | > 100 | 50 - 100 | > 100 | 50 - 100 |
| **8f** | 10 - 50 | > 100 | 10 - 50 | > 100 | 50 - 100 |
| **8g** | 5 - 10 | 5 - 10 | 5 - 10 | > 100 | 10 - 50 |
| **8h** | 5 - 10 | > 100 | 50 - 100 | > 100 | > 100 |
| **8i** | > 100 | > 100 | 10 - 50 | > 100 | 50 - 100 |
| **8j** | 10 - 50 | > 100 | > 100 | > 100 | > 100 |
| **8k** | > 100 | > 100 | 50 - 100 | > 100 | 50 - 100 |
| **8l** | 5 - 10 | 5 - 10 | 5 - 10 | > 100 | 5 - 10 |
| **8m** | 50 - 100 | > 100 | 50 - 100 | > 100 | 50 - 100 |
| **8n** | 5 - 10 | > 100 | 5 - 10 | 5 - 10 | > 100 |
| **8o** | 10 - 50 | 10 - 50 | 50 - 100 | > 100 | > 100 |
| **8p** | 50 - 100 | 50 - 100 | 10 - 50 | > 100 | 50 - 100 |
| **8q** | 5 - 10 | 50 - 100 | 50 - 100 | 5 - 10 | > 100 |
| **8r** | 5 - 10 | 50 - 100 | 50 - 100 | 10 - 50 | > 100 |
| **5-Fluorouracile** | 3.36 | 8.17 | 0.95 | 8.46 | 1.77 |
| **Control CI[g]** | (2.69 - 4.15) | (7.20 - 9.25) | (0.77 - 1.15) | (6.80 - 10.54) | (1.64 - 1.92) |
| **Etoposide** | 0.49 | 0.52 | 1.99 | 2.37 | 0.29 |
| **Control CI[g]** | (0.33 - 0.71) | (0.50 - 0.55) | (1.33 - 2.86) | (1.93 - 2.89) | (0.25 - 0.51) |

[a] $IC_{50}$ values (μM): drug concentration responsible for the inhibition of 50% of the growth of the specified cell line after 72 h; [b] Human lung cancer line; [c] Human glioma cancer line; [d] Human breast cancer line; [e] Human melanoma cancer line; [f] Mouse melanoma cancer line; [g] CI for confidence interval

This study may provide valuable information for further design of pyrazolo[3,4-*d*]thiazoles and pyrazolo[3,4-*c*]-pyrazoles containing mono-substituted aromatic phenyl groups as potential anti-tumour agents. It is worth

noting that the presence of both *p*-$CF_3$- and *p*-$CH_3O$-groups on the aromatic ring seems to affect anti-tumour activity of certain herein studied heterobicycles.

*Determination of in vitro cytotoxic activity*

The growth level of four cancer cell lines was determined using a colorimetric MTT assay. Cancer cell lines and growth medium were obtained from CLS Cell Line Service GmbH. Human skin melanoma SK-MEL-28, mouse melanoma skin B16-F1 and human brain glioma HS-683 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, 2 mM *L*-glutamine, and 10% foetal bovine serum (FBS). The human lung carcinoma cell line A-549 was grown in DMEM/Ham's F12 (1:1) supplemented with 2 mM *L*-glutamine and 5% FBS and human breast adenoma-carcinoma MCF-7 in Eagle's minimum essential medium supplemented with 2 mM *L*-glutamine, sodium pyruvate, nonessential amino acid, 10 μg/mL human insulin and 10% FBS. MTT assay is based on the reduction of the yellow product MTT to purple-blue formazan by mitochondrial dehydrogenase of metabolically active cells. The number of living cells after incubation in the presence (or absence, control) of the tested molecule is directly proportional to the blue colour, which was measured by spectrophotometry. Briefly, cells were seeded (100 μL of a $2.5 \times 10^4$ cells/mL suspension) in 96-well culture plates (Nunc™ Edge 2.0, Fisher) and incubated for 24 h. Each compound (starting from DMSO solutions, stable for months) was assessed in serial dilution (four concentrations in 0.1% DMSO at the highest concentration) in three replicates (n = 3) and incubated for 72 h. Thereafter, MTT (5 mg/mL solution in phosphate-buffered saline) was added to each well (10% v/v) and cells were further incubated for 4 h. Then, after removing the culture medium, the blue crystals were dissolved in 100 μL of 100% DMSO and absorbance measured at 540 nm using a 620 nm reference. The absorbance of the serial dilution of each cell line treated under the same conditions, but without the tested compounds was measured to generate a standard curve allowing $IC_{50}$ determination ($IC_{50}$ is defined as the concentration reducing cell growth by 50%).

## Conclusions

To conclude, we describe herein a route towards substituted and condensed poly-heterocycles and their biological evaluation. The original scaffolds were obtained by relying on a CAN deprotection strategy. Two series of analogues (pyrazolo[3,4-*d*]thiazoles and pyrazolo[3,4-*c*]pyrazoles) were tested for their cytotoxic activities against one mouse and four human cancer cell lines.

*In vitro* bioassays proved that pyrazolo[3,4-*d*]thiazole containing both trifluoromethyl and methoxy group on the phenyl rings (compound **3l**) exhibited good to excellent cytotoxic activities against HS-683 ($IC_{50}$ = 0.1 - 1 μM) and A-549 ($IC_{50}$ = 1 - 10 μM). Presence of cyano and/or methoxy substituents on the phenyl rings attached to the pyrazolo[3,4-*c*]pyrazoles showed encouraging anti-tumour effect against A-549 ($IC_{50}$ = 1 - 10 μM as compounds **8g**, **8l**, **8n**), HS-683 ($IC_{50}$ = 1 - 10 μM as compounds **8g**, **8l**), MCF-7 ($IC_{50}$ = 1 - 10 μM as compounds **8g**, **8l**, **8n**), SK-MEL-28 ($IC_{50}$ = 1 - 10 μM as compound **8n**) and B16-F1 ($IC_{50}$ = 1 - 10 μM as compound **8l**).

The results obtained are promising, and further studies on the structural optimization of methoxy, trifluoromethyl and cyano substituted phenyl heterobicycles are well underway, targeting on how to improve their anti-tumour potency.

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## Conflict of interest

The authors declare no conflict of interest.

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