EVALUATION OF THE ANTI-PROLIFERATIVE PROFILE OF A NEW (4-BROMOPHENYL)-1H-PYRAZOLE DERIVATIVE*

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Abstract. Using the aminopyrazole scaffold, N-[5-(4-bromophenyl)-1*H*-pyrazol-3--yl]carbamothioyl]benzamide was synthesized as a new anti-proliferative agent, hypothesized to target as a specific molecular substrate, several protein kinases. This compound (PRZ) showed anti-proliferative and cytotoxic effects in various human cancer cell lines. We assessed the effect of PRZ, along with the effects of cisplatin (CPT), methotrexate (MTX) and of their binary combinations with PRZ on *Triticum aestivum root* elongation test. We assessed phenomena such as antagonism or synergism, for a better understanding of the mechanisms underlying the anti-proliferative effect of PRZ. MTX and, to a lesser extent, PRZ inhibited cell growth and differentiation. More significant, their equimolar mixture had a more intense effect than MTX alone, but lower than the sum of the effects of each individual compound. The partial additive effect suggests a related mechanism of action for the two compounds. No significant effect was noticed for cisplatin alone, or for its association with PRZ.

Key words: pyrazolylthiourea, root elongation, *Triticum aestivum*, methotrexate, cisplatin.

INTRODUCTION

The pyrazole ring represents an important tool in the design of specific anticancer therapies, various types of pyrazole derivatives being described as anti-proliferative agents [1]. Ruxolitinib is a pyrazole derivative used as Janus kinase inhibitor in various types of cancer [5]. Crizotinib is an anti-cancer pyrazole derivative, which acts as anaplastic lymphoma kinase and c-ros oncogene 1 inhibitor, used for the treatment of non-small cell lung carcinomas [13]. Ilorasertib is a pan inhibitor of Aurora kinases [8], vascular endothelial growth factor and

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platelet-derived growth factor receptors, useful in solid and haematological cancers [2].

New potential anti-proliferative agents targeting kinases were developed using aminopyrazole as starting point, N-[5-(4-bromophenyl)-1*H*-pyrazol-3-yl]carbamothioyl]benzamide emerging as an promising lead with very good anti-proliferative and cytotoxic effects in NCI-60 human cancer cell lines [6, 9]. At 10 μM/L, it inhibits completely the growth of HCT-116 colon cells and up to 90% the growth of lung cells NCI-H522 and of glioblastoma cell line U251. The best anti-proliferative effects were recorded on the renal cancer cells with *pIC*50 values ranging from 4.40 to 5.50 [6].

The aim of this study is using the *Triticum aestivum* root elongation test for a better understanding of the anti-proliferative pathways activated by the above-mentioned compound. For this purpose, we also investigated the effects of cisplatin (CPT), methotrexate (MTX) and of their binary combinations with N-[5-(4-bromophenyl)-1*H*-pyrazol-3-yl]carbamothioyl]benzamide (PRZ) on *Triticum aestivum root* elongation test, assessing potential phenomena such as antagonism or synergism.

The inhibition of the root growth and the observation of the cytological parameters is a simple and efficient method to evaluate new potential anticancer agents and it can provide useful information about the genotoxicity risks [7, 10]. The use of phytobiological tests has major advantages such as quicker outcome, simplified operative procedure and lower costs [15].

The anti-proliferative effect of methotrexate is a consequence of the inhibition of dihydrofolate reductase activity resulting in a reduced production of nucleotides. In addition to the effects on nucleotide biosynthesis, methotrexate has been linked to a decrease in cellular methylation, blocking the remethylation of homocysteine [16]. This results in the accumulation of a potent product inhibitor of cellular methyltransferases, which also represents a mechanism for the anti-proliferative effect of antifolates. One major class of methylated proteins is the CaaX-type prenyl proteins, including the Ras family of GTP-binding proteins. Ras is a central component in many signal transduction pathways, including pathways important for cell growth and differentiation. Methotrexate has therefore an additional mechanism of action inhibiting in Ras signaling [16].

The cytotoxicity of cisplatin is primarily a result of its interaction with nucleophilic N7-sites of purine bases in DNA to form DNA–protein and DNA–DNA inter- and intra-strand crosslinks [14], thus activating apoptosis via p53 and MAPK [4].

MATERIALS AND METHODS

COMPOUND SYNTHESIS

Principle

The pyrazole lead, N-[[5-(4-bromophenyl)-1*H*-pyrazol-3-yl]carbamothioyl] benzamide was synthesized by treating benzoyl chloride with ammonium isothiocyanate to give benzoyl isothiocyanate, which reacted with 5-(4-bromophenyl)-1*H*-pyrazol-3-amine.

Materials

All starting materials, reagents, and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Acetone was dried over 3 Å molecular sieves and distilled before use.

Method

The pyrazole derivative was synthesized using the method described in [7], with some modifications. The raw obtained benzoyl chloride (20 mmol) is dissolved in acetone (20 mL) and added to a solution of ammonium thiocyanate (20 mmol) and PEG-400 (0.1 mL) dissolved in acetone. The ammonium chloride is removed by filtration and 5-(4-bromophenyl)-1*H*-pyrazol-3-amine (10 mmol) is added. The mixture is heated for one hour and then poured with stirring into 100 mL of 0.1 M hydrochloric solution and the resulting precipitate is separated by filtration.

TRITICUM AESTIVUM ASSAY

MTX, CPT and the pyrazole derivative PRZ were dissolved in DMSO and diluted with distilled water to obtain solutions with concentration of 1, 0.5, 0.1, 0.05 and 0.01 μM/L. The binary equimolar combinations MTX-PRZ and CPT-PRZ were prepared for the above mentioned concentrations.

Dry caryopses of *Triticum aestivum* (Boema cultivar), supplied by SC Adaflor SRL (Tulcea, Romania), were soaked for 24 h in distilled water. A number of 20 caryopses were equally distributed on filter paper disks in Petri dishes of 90 mm diameter and treated with 5 mL of each test solution. The bioassay was conducted in a plant growth chamber (Sanyo MLR-351 H, USA) at 25 ± 1 ˚C, 75% relative humidity in the absence of light [12]. A negative control sample (C) was prepared with distilled water. Germination was monitored after 24 h and the radicle length was measured with the application Image J version 1.46 r (Wayne Rasband National Institutes of Health, USA). The values of root elongation were expressed in mm.

D'Agostino Pearson normality test ($\alpha = 0.5$) was performed in order to determine the normal distribution of root elongation values.

The inhibitory effect (I) induced by each compound was calculated with Microsoft Office Excel 2003 (Microsoft Corp., USA), using the following equation:

$$I = 100 - \frac{R}{C} \times 100 \quad (1)$$

where: R – sample average of the root elongation (mm); C – control sample average of the root elongation (mm); 100 – the results are expressed as a percentage.

Due to abnormal distribution of the values of the radicular elongation, a non-parametric analysis (Kruskall Wallis test, post test Dunn) was performed.

Least square method was applied in order to plot the dose-inhibitory effect curves and to calculate the $IC50$ parameter, representing the concentration at which root elongation is inhibited by 50%, and goodness of fit (r^2) [3, 11]. The determinations were performed using the GraphPad Prism v.5.0 software (GraphPad Software, USA).

RESULTS AND DISCUSSION

The highest inhibition was induced by MTX, followed by its combination with PRZ and then by PRZ alone. CPT did not produce any significant modification on root elongation, whereas its combination with PRZ induced a slight inhibition.

Table 1

The results of D'Agostino Pearson normality test on distribution of the root elongation values

Concentration (µM)	**1**	**0.5**	**0.1**	**0.05**	**0.01**
Sample	***p* value**				
PRZ	0.0141	0.8765	0.0002	0.5019	0.0014
MTX	0.7976	0.3625	< 0.0001	0.0013	0.9614
MTX-PRZ	0.9130	0.6786	< 0.0001	0.3244	0.4325
CPT	0.0004	0.6649	0.0013	0.5995	< 0.0001
CPT-PRZ	0.1310	0.1594	0.1474	0.0057	0.0782
Control	0.0459				

Normal distribution was considered for $p > 0.05$.

As shown in Table 1, Gaussian distribution was obtained for root elongation values of wheat embryonic roots treated with: PRZ at 0.05 and 0.5 µM; MTX at 0.01 and 0.5 µM; MTX-PRZ at 0.01, 0.05, 0.5 and 1 µM; CTP at 0.05 and 0.5 µM; CTP-PRZ at 0.01, 0.1–1 µM.

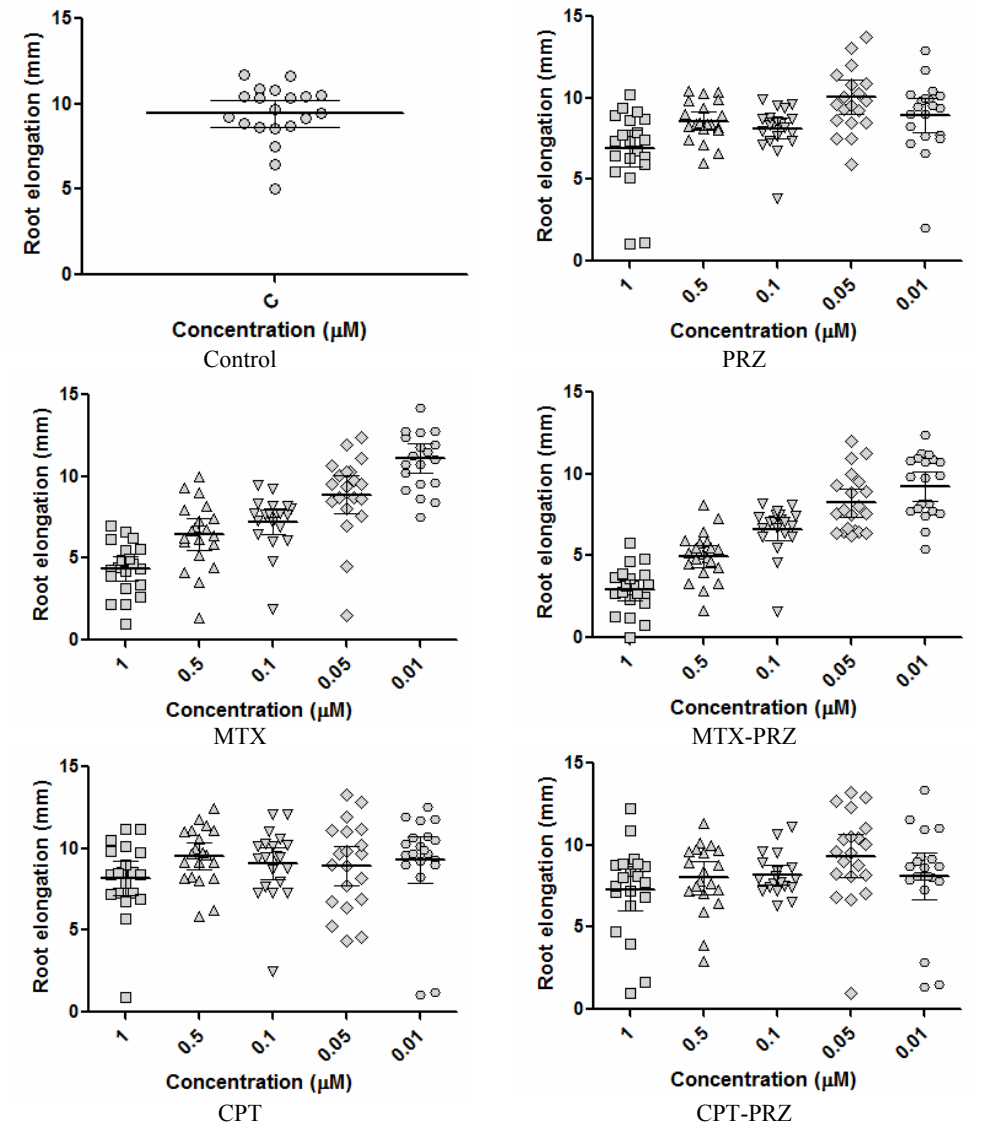


Fig. 1. Root elongation values obtained following the treatment with MTX, CPT, PRZ and their equimolar combinations at concentrations ranging from 1 to 0.01 µM; horizontal line represents the average value and the error bars are representing the upper and lower limits of the 95% confidence interval of the average.

The distribution of the root elongation values measured after 24 h treatment with serial dilution of PRZ, MTX, CPT and their binary mixture is presented in Figure 1 along with the control group.

Following D'Agostino Pearson normality test results, Kruskal Wallis and Dunn tests ($\alpha = 0.05$) were applied in order to evaluate the differences between the effect induced by the tested compounds. The tests were applied in order to assess the differences between control and all samples and between single compounds and their combinations. Thus, at the analysis of the root elongation of PRZ and C, Kruskal Wallis test revealed statistical differences and Dunn test emphasized differences only at concentrations 0.1–1 μM. At the analysis of MTX and C, Kruskal Wallis test was significant ($p < 0.0001$), and Dunn test revealed statistical significance only at concentrations 0.1, 0.5 and 1 μM. Similar results were obtained for the combination MTX-PRZ. At the comparison of the results obtained for MTX, PRZ and their combination, statistical differences were obtained only for pair PRZ *vs*. MTX-PRZ.

Table 2

The assessment of inhibitory effect

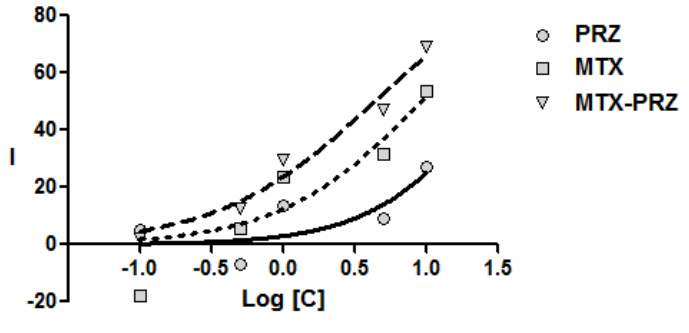
Sample	***IC*50 (μM)**	**Goodness of fit (r^2)**
PRZ	29.01	0.7078
MTX	9.19	0.8162
MTX-PRZ	4.37	0.9692
CPT	–[a]	–[a]
CPT-PRZ	–[a]	–[a]

[a] – *IC*50 and r^2 could not be determined.

At the analysis of the pairs PRZ, CPT and CPT-PRZ, statistical differences were registered by applying Kruskal Wallis test ($p < 0.001$). Dunn test revealed slighty differences ($p \leq 0.5$) only at the concentrations 0.01 and 0.5 μM, and only for the pair CPT *vs.* CPT-PRZ.

Dunn test suggested PRZ induces a similar inhibitory effect in both associations with MTX and CPT, but the concentration-inhibitory effect curves revealed a partially additive effect for the association PRZ-CPT (the effect was lower than that induced only by PRZ (Fig. 2).

The additive effect noticed for the association PRZ-MTX is also sustained by the *IC*50 values. Thus, the highest *IC*50 was calculated for PRZ, followed by MTX and then their combination, corresponding to their decreasing order of inhibition. For the mixture of PRZ and CPT, a moderate inhibitory effect was observed, being lower that the effect induced by PRZ alone.



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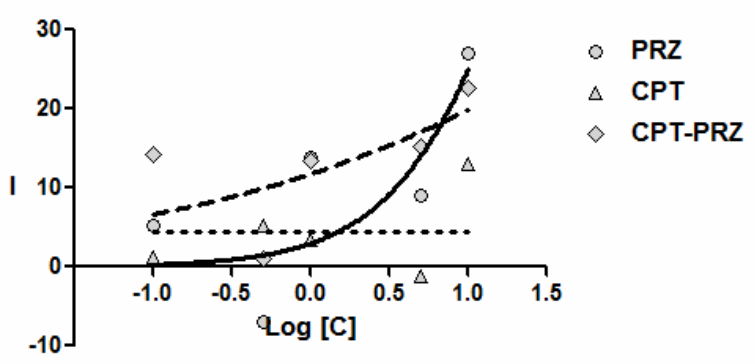

B

Fig. 2. The concentration-inhibitory effect curves for PRZ, MTX, CPT and their combinations; the concentration is expressed as μM.

CONCLUSIONS

The results of our study confirmed the anti-proliferative effects of the lead pyrazole derivative PRZ. The equimolar mixture of PRZ and MTX presented superior growth inbitory effects comparing to MTX alone, but lower than the sum of the effects of each individual compound. This partially additive effect suggests a related mechanism of action for the two compounds.

The study demonstrated the usefulnes of the Triticum aestivum root elongation test, as a simple method to better understand the mechanisms underlying the anti-proliferative effect of new compounds by observing antagonism or synergism phenomena in association with standard cytotoxic drugs.

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