

Hematological effects of protein kinases inhibitor maleimide derivative (1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione)

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Abstract: Maleimide derivative (MD, 1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione) is an inhibitor of a number of protein kinases and proliferative activity of tumour cells. A study of potential anticancer drug effects on different systems of the organism, including the hemopoietic system, is an urgent issue. The aim of the present study was to investigate MD effects on rat blood cells parameters. It was found that daily treatment with MD for 25 days (2.7, 0.027, 0.00027 mg/kg) does not significantly affect the erythroid parameters of rats. However, MD at 2.7 and 0.027 mg/kg slightly increased and at 0.00027 mg/kg decreased the erythrocytes mean volume. MD at 0.00027 mg/kg did not affect the number of blood leukocytes. MD at 2.7 and 0.027 mg/kg reduced the leukocytes count by decreasing the absolute number of the neutrophilic and eosinophilic granulocytes and monocytes. Thus, the MD-induced effects on the erythrocytes and thrombocytes parameters do not restrict its application as an antitumor drug in indicated doses. However, it is necessary to control the leukocytes count to detect the occurrence of leukopenia during MD therapy.

Key words: maleimide derivative, erythrocytes, leukocytes, thrombocytes

INTRODUCTION

Changes of the regulatory protein kinases activity in the cells may evoke uncontrolled proliferation and as a result the development of oncological disorders [1]. The revealing of mutated protein kinases in numerous tumours stimulated the search for new medicinal substances to inhibit these protein kinases and suppress tumour growth. Successful application of the tyrosine kinase inhibitor imatinib for the treatment of chronic myeloid leukemia was a starting point for development of the target therapy as a promising theme in oncology. The search for monoclonal antibody (mAb) or small molecules blocking kinase-substrate interaction, or its adenosine triphosphate binding site, resulted in the creation of drugs such as: trastuzumab (mAb) for human epidermal growth factor, receptor 2 (HER2-protein) for the treatment of breast cancer, bevacizumab (mAb for vascular endothelial growth factor A) for the treatment of metastatic colorectal cancer, gefitinib (inhibitor of tyrosine kinase domain for the epidermal growth factor receptor) for treatment of lung and colorectal cancer, etc. Such target therapy has a number of advantages including high specificity and low side effects (it does not affect normal proliferating cells) [2].

The inhibitor of some protein kinases, YES (Yamaguchi sarcoma viral oncogene homolog 1), Src(h) (Rous sarcoma oncogene cellular homolog), ZAP70 (Zeta-chain-associated protein kinase 70), Syk(h) (Spleen tyrosine kinase), PDK1

(3-phosphoinositide-dependent kinase 1), etc. Maleimide derivative (MD) (1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione) (fig.1) was synthesized by scientific production at the Chemical-Biological Centre of Taras Shevchenko National University in Kiev [3, 4].

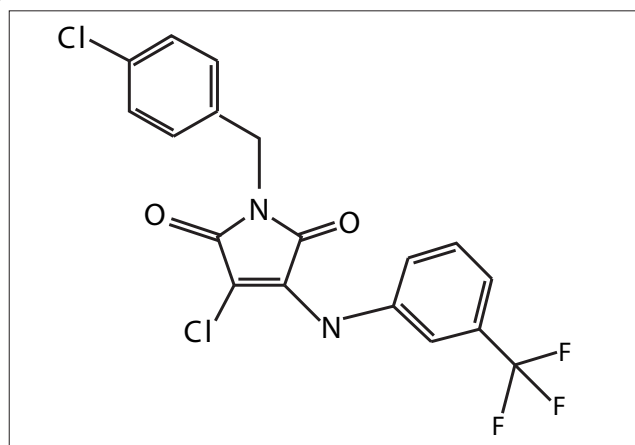


Figure 1 Maleimide derivative 1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione.

It was shown that MD in the concentration of 100 μM inhibited proliferation of human tumour cells *in vitro* (breast cancer, non-small cell lung cancer and neuroblastoma cell lines) by more than 68% [5]. Also, MD suppressed the growth of cancer cells (human lung adenocarcinoma epithelial cells line A-549, the line of the cells resistant to interferon in the concentration of 1,0000 MO/ml A-549-R, human colonic adenocarcinoma cell line SW-620). The growth of cell line SW-620 and line of

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the cells resistant to interferon were the most sensitive to MD. LD₅₀ of MD for the white outbred rats is 500 mg/kg (per os). This is why MD is a slightly toxic substance, belonging to third (from 151 to 5,000 mg/kg) class hazard) [4].

Thus, MD is a potential compound for application in clinical practice. It may be used for the treatment of oncological disorders, especially of the gastrointestinal tract, and in the case of tumours resistant to interferon.

The protein kinases YES, Src(h), ZAP70, Syk(h), PDK1, etc., play an important role in the proliferation, differentiation and functioning of normal cells, including hemopoietic cells [6-8]. Therefore, the application of the inhibitor of these protein kinases may affect blood cells production, differentiation and functions. Thus, a study of potential anticancer drug effects on different systems of the organism, including the hemopoietic system, is an urgent issue. The aim of the present study was to investigate the effects of MD (1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione) on standard blood cells parameters in healthy rats.

MATERIALS AND METHODS

The experimental design was approved by the Animal Ethics Committee of Taras Shevchenko National University in Kiev. Experiments were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the use and care of experimental animals. The experiments were carried out on 20 white outbred male rats (130-160 g). The animals were kept in colony cages with free access to food and tap water, under standardized housing conditions. The rats were divided into 4 groups: a control group and 3 experimental groups which were treated daily with MD (1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione) for 25 days, respectively, in the doses 0.00027, 0.027 and 2.7 mg/kg dissolved in 0.2 ml of sunflower oil, *per os*. The control group was given 0.2 ml sunflower oil. The doses of MD were selected according to their effectiveness against tumour cells proliferation in cell culture studies. MD at 0.00027 mg/kg corresponds to the minimal inhibitor activity against cell proliferation, at 0.027 mg/kg – 50% inhibitor activity (IC₅₀), at 2.7 mg/kg – maximal inhibitor activity (90%) [4, 5].

The effects of MD were tested at different doses on blood cells parameters in healthy rats. The blood was collected into test tubes with EDTA the day after the last administration of MD. The quantitative blood parameters, involving the number of erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), coefficient of variation of the red cell volume distribution width (RDW-CV), number of leukocytes, number of platelets, mean platelets volume (MPV), and platelets distribution width (PDW) were

determined using cells counter Sysmex F-800. Differential analysis of leukogram was performed by Pappenheim's blood smears staining, calculating 200 cells.

The data were statistically processed using one-way ANOVA followed by the *post-hoc* Dunnett's test using SPSS 16.0 for Windows. Regression analysis was used for study of dose-response effect of maleimide derivative on blood cells.

RESULTS

Administration of MD at 0.00027, 0.027 or 2.7 mg/kg for 25 days did not affect the parameters of red blood cells in rats: the number of erythrocytes, hematocrit, hemoglobin, MCH, MCHC, RDW-CV. However, one-way ANOVA test showed MD effect on MCV ($p < 0.01$). The analysis of differences in experimental groups *vs.* control group (Dunnett's test) revealed that MD at 0.00027 mg/kg slightly reduced MCV ($p = 0.07$), but at 0.027 and 2.7 mg/kg tended to increase it ($p = 0.2$), but the difference was not significant in both cases. Since increasing the MCV was common for 0.027 and 2.7 mg/kg experimental groups we combined data from these groups into a single one to confirm these changes. ANOVA ($p < 0.01$) and Dunnett's tests ($p = 0.08$) confirmed the trend of MCV increasing in experimental groups treated with MD at 0.027 and 2.7 mg/kg. The regression analysis showed positive a dose-dependent effect on MCV with coefficient determination $R = 0.66$ (R Square = 0.43).

Treatment with MD for 25 days did not affect the leukocytes count in any of the experimental groups. However, one-way ANOVA test showed a trend towards reduction of total leukocytes count ($p = 0.051$), eosinophilic ($p < 0.05$) and neutrophilic ($p = 0.09$) granulocytes and monocytes ($p = 0.1$). Analysis of individual data showed that MD at 0.027 and 2.7 mg/kg reduced leukocytes count in more than 50% of rats *vs.* control group. As a confirmation of present data, we found reduced leukocytes count after treatment with MD at 5 mg/kg (data not shown). Since the leukocytes level is an important parameter of the organism condition, we combined data from experimental groups treated with MD at 0.027 and 2.7 mg/kg into a single group for MD effect analysis. One-way ANOVA test confirmed the influence of MD on total leukocytes count ($p < 0.05$), eosinophilic ($p < 0.01$) and neutrophilic ($p < 0.05$) granulocytes and monocytes ($p < 0.05$). Reduction of these parameters in the combined group *vs.* the control group was confirmed by Dunnett's test ($p < 0.05$). Regression analysis showed a dose-dependent effect of MD on total leukocytes count (coefficient determination $R = 0.59$, R Square = 0.35), eosinophilic granulocytes ($R = 0.76$, R Square = 0.58) and monocytes ($R = 0.50$, R Square = 0.25).

MD at 2.7, 0.027 and 0.00027 mg/kg did not affect thrombocytes parameters: platelet number, MPV and PDW (Table 1).

Table 1 Effects of maleimide derivative on rat blood cells parameters, \pm SEM (per os, for 25 days).

Group	Leukocytes, $\times 10^9/l$	Red blood cells, $\times 10^{12}/l$	Hemo-globin g/l	Hematocrit %	MCV, fl	MCH, pg	MCHC, g/l	RDW-CV, %	Platelets, $\times 10^9/l$	PDW, fl	MPV, fl
Control group	13.87 \pm 0.67	6.30 \pm 0.19	120.5 \pm 2.59	33.05 \pm 1.06	52.45 \pm 0.49	19.15 \pm 0.26	365.3 \pm 5.83	18.52 \pm 0.50	736.7 \pm 69.65	8.62 \pm 0.18	8.10 \pm 0.08
0.00027mg/kg	14.70 \pm 0.49	6.39 \pm 0.18	114.3 \pm 3.84	32.00 \pm 0.51	50.13 \pm 0.67	17.93 \pm 0.97	357.7 \pm 15.34	16.83 \pm 0.93	681.0 \pm 41.02	9.13 \pm 0.75	8.47 \pm 0.57
0.027 mg/kg	10.43 \pm 2.00	6.44 \pm 0.42	118.8 \pm 5.12	34.70 \pm 1.87	54.08 \pm 1.01	18.70 \pm 1.34	345.5 \pm 22.69	18.73 \pm 0.37	787.7 \pm 34.97	9.03 \pm 0.89	8.30 \pm 0.42
2.7 mg/kg	10.20 \pm 1.78	5.88 \pm 0.16	114.2 \pm 3.64	31.72 \pm 0.94	53.90 \pm 0.39	19.42 \pm 0.52	360.6 \pm 10.70	18.60 \pm 0.41	705.4 \pm 32.35	8.78 \pm 0.23	8.22 \pm 0.15

MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; RDW-CV – coefficient of variation of red cell volume distribution width; MPV – mean platelets volume; PDW – platelets distribution width.

Table 2 Effects of maleimide derivative on rat leukogram parameters, \pm SEM (per os, for 25 days).

Group	Band neutrophils,		Segmented neutrophilic granulocyte		Lymphocytes		Monocytes		Eosinophilic granulocytes	
	%	$\times 10^9/l$	%	$\times 10^9/l$	%	$\times 10^9/l$	%	$\times 10^9/l$	%	$\times 10^9/l$
Control group	0.92 \pm 0.33	0.13 \pm 0.05	18.92 \pm 2.63	2.65 \pm 0.47	69.42 \pm 3.07	9.56 \pm 0.41	7.08 \pm 0.85	1.00 \pm 0.17	3.67 \pm 0.53	0.50 \pm 0.07
0.00027 mg/kg	0.33 \pm 0.29	0.05 \pm 0.05	13.00 \pm 1.80	1.91 \pm 0.32	76.00 \pm 2.63	11.19 \pm 0.76	6.00 \pm 1.52	0.87 \pm 0.24	4.67 \pm 0.58	0.68 \pm 0.08
0.027 mg/kg	0.38 \pm 0.24	0.03 \pm 0.02	13.75 \pm 1.31	1.41 \pm 0.14	77.25 \pm 2.83	8.09 \pm 1.04	5.75 \pm 1.05	0.59 \pm 0.09	2.88 \pm 0.83	0.31 \pm 0.10
2.7 mg/kg	0.70 \pm 0.34	0.06 \pm 0.03	18.80 \pm 3.37	1.71 \pm 0.14	71.60 \pm 4.26	7.58 \pm 1.69	5.60 \pm 0.91	0.54 \pm 0.09	3.20 \pm 0.78	0.30 \pm 0.06

Thus, MD-induced effects on the erythrocytes and thrombocytes parameters do not restrict its application as an anti-tumour drug at the indicated doses. However, it is necessary to control the leukocytes count to detect the occurrence of leukopenia during MD therapy.

DISCUSSION

The main purpose of the pre-clinical studies was to obtain preliminary efficacy, toxicity, safety and pharmacokinetic information on the studied substance *in vitro* and *in vivo*. Determination of the toxicity of the studied chemical substances is a very important and responsible stage of the pre-clinical studies. The choice of the medicinal doses, treatment regime, therapy strategy and effectiveness will depend on that.

Hemopoietic tissue belongs to the highly proliferative tissues. The pool of the hemopoietic precursor cells and the mature blood elements are in dynamic equilibrium where the death and destruction of mature cells is balanced by constant production and entry of the cells into the blood. On average, from 200-400 million blood cells are destroyed and formed during 24 hours in an adult organism. The destructive impact on the hemopoiesis is accompanied by cell death, cell differentiation and maturation disorder, activation of the stem cells which under normal conditions are non-activated.

Studies of the effects of protein kinase inhibitors MDs on blood cells are scarce. For instance, it is known that eosin-5-maleimide is bound with erythrocyte membrane proteins of band 3 and band 4.1 [9], and that feature is used to diagnose hereditary disorder of the erythrocyte membranes [10].

MD is specific inhibitor of PDK1 (phosphoinositide-dependent) serine-threonine protein-kinase that phosphorylates a number of other protein kinases (e.g. A, G, C, B) which are important for hemopoietic cells differentiation [6, 11]. Protein kinase C is known to play a significant role in erythrocytes functions [12]. Activation of protein kinase C in erythrocytes causes exposure phosphatidylserine, cell shrinkage and suicidal death of erythrocytes (eryptosis) [13]. Staurosporine, known as a natural inhibitor of the protein kinase C, prevents the appearance of phosphatidylserine in the outer layer of the erythrocyte plasma membrane [14]. Phagocytosis of erythrocytes by macrophages occurs via activation of protein kinase B/Akt, protein kinase C and phosphatidylinositol-3-kinase [15]. Thus, protein kinase C plays a significant role in erythrocytes differentiation, function, and death. MD may affect protein kinase C via PDK1-protein kinase. The changes of erythrocyte volume obtained in our study may be connected with the changes in protein kinase C activity.

N-ethylmaleimide is a thio-alkylating agent causing an increase in the internal concentration of Ca^{2+} ions by their

entry into the rat neutrophils from the external medium [16]. Therefore, an increase in Ca^{2+} ions in cytosole affects both functional activity of neutrophilic granulocytes and apoptosis of these cells [17, 18]. Activation of neutrophilic granulocytes with following bacteria absorption is accompanied by Syk-kinase activation [19]. Src, Syk, PI3-kinase and Erk suppress spontaneous apoptosis of neutrophilic granulocytes [20]. Since the MD is an inhibitor of Src, Syk, PDK1 kinases, the tendency revealed in the present study to change the number of neutrophilic granulocytes in the blood might be connected to apoptosis activation.

The decreased number of monocytes after MD treatment shown in our study might be related to PDK1 inhibition which is necessary for monocytes differentiation [21]. Moreover, Syk, Src-related kinases and PI3-kinase (activate PDK1) play important roles in monocytes and macrophages phagocytosis [25], and their inhibition may impact on this function and needs further investigation.

Other maleimide derivatives (1-[6-[[17beta-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]1H-pyrrole-2,5-dione, inhibitor of phospholipase C) inhibit the transfer of phospholipids from very low density lipoproteins (VLDL) to thrombocytes during their activation [22]. In our study, MD had neither quantitative nor qualitative effects on thrombocytes parameters. Thus, the effects of MD on the activity of these cells needs further investigation.

Thus, administration of MD for 25 days in the doses of 2.7, 0.027, 0.00027 mg/kg showed an insignificant impact on the rat blood parameters, which does not restrict application of this compound as a potential anti-tumour drug.

CONCLUSIONS

1. Administration of MD (1-(4-Cl-benzyl)-3-Cl-4-(CF₃-phenylamino)-1H-pyrrole-2,5-dione) at 2.7, 0.027, 0.00027 mg/kg does not affect the parameters of the erythrocytic lineage, *viz.*, erythrocytes number, hemoglobin, hematocrit, mean hemoglobin content in erythrocyte, mean hemoglobin concentration in erythrocyte, or the coefficient of variation of the red cell volume distribution width. MD at 2.7 and 0.027 mg/kg slightly increased and at 0.00027 mg/kg decreased the erythrocytes mean volume.
2. MD at 0.00027 mg/kg does not affect the blood leukocytes number, but at 0.027 and 2.7 mg/kg influences the total leukocytes count, eosinophilic and neutrophilic granulocytes and monocytes. These parameters should be monitored under MD application.
3. Treatment with MD (2.7, 0.027 and 0.00027 mg/kg) for 25 days does not affect thrombocytes parameters, *viz.*, thrombocytes count, mean volume and volume distribution width.

REFERENCES

1. Novak K: Conference report – Protein kinase inhibitors in Cancer – Mixing and Matching. *Med Gen Med* 2004, **6**, 23.
2. Zeng Z, Samudio IS, Zhang W, Estrov Z, Pelicano H, Harris D, Frolova O, Hail N, Chen W, Kornblau SM, Huang P, Lu Y, Mills GM, Andreeff M, Konopleva M: Simultaneous Inhibition of PDK1/AKT and Fms-Like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and Apoptosis in Acute myelogenous leukemia. *Cancr Res* 2006, **66**, 3737-3746.
3. Dubinina GG, Chupryna OO, Platonov MO, Borysko PO, Ostrovska GV, Tolmachov AO, Shtil AA: *In Silico* design of protein kinase inhibitors: successes and failures. *Anticancer Agents Med Chem* 2007, **7**, 171-188.
4. Dubinina GG, Volovenko YuM: Compound of 1,4-disubstituted 5-amino-1,2-dihydropyrrole-3-one having anticancer activity. Pat. 22204 (UA), 21.02.2006. Appl. U200601855. 25.04.2007.
5. Dubinina GG, Golovach SM, Kozlovsky VO, Tolmachov AO, Volovenko YuM: Antiproliferative activity of the new derivatives of 1-(4-R-benzyl)-3-R1-4-(R2-phenylamino)-1-pyrrole-2,5-dione. *Journal Organichnoi ta farmacevtichnoi khimii* (Ukraine) 2007, **5**, 39-49.
6. Bone HK, Welham MJ: Phosphoinositide 3-kinase signalling regulates early development and developmental haemopoiesis. *J Cell Sci* 2007, **120**, 1752-1762.
7. Shrivastav A, Varma S, Lawman Z, Yang SH, Ritchie SA, Bonham K, Singh SM, Saxena A, Sharma RK: Requirement of N-myristoyltransferase 1 in the development of monocytic lineage. *J Immunol* 2008, **180**, 1019-1028.
8. Rane SG, Reddy EP: JAKs, STATs and Src kinases in hematopoiesis. *Oncogene* 2002, **21**, P. 3334-3358.
9. King MJ, Smythe JS, Mushens R: Eosin-5-maleimide binding to band 3 and Rh-related proteins forms the basis of a screening test for hereditary spherocytosis. *Biochem Biophys Res Commun* 2003, **312**, 467-472.
10. Kedar PS, Colah RB, Kulkarni SE, Ghosh K, Mohanty D: Experience with eosin-5-maleimide as a diagnostic tool for red cell membrane cytoskeleton disorders. *Bioconj Chem* 2003, **14**, 1253-1259.
11. Yang KJ, Shin S, Piao L, Shin E, Li Y, Ah Park K, Byun HS, Won M, Hong J, Kweon GR, Hur GM, Seok JH, Chun T, Brazil DP, Hemmings BA, Park J: Regulation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) by Src involves tyrosine phosphorylation of PDK1 and Src SH2 domain binding. *J Biol Chem* 2008, **283**, 1480-1491.
12. De Jong K, Rettig MP, Low PS, Kuypers FA: Protein kinase C activation induces phosphatidylserine exposure on red blood cells. *Biochemistry* 2002, **41**, 12562-12567.
13. Niemoeller OM, Bentzen PJ, Lang E, Lang F: Adenosine protects against suicidal erythrocyte death. *Pflugers Arch* 2007, **454**, 427-439.
14. Klarl BA, Lang PA, Kempe DS: Protein kinase C mediates erythrocyte 'programmed cell death' following glucose depletion. *Am J Physiol Cell Physiol* 2006, **290**, C244-C253.
15. Lutz MA, Correll PH: Activation of CR3-mediated phagocytosis by MSP requires the RON receptor, tyrosine kinase activity, phosphatidylinositol 3-kinase, and protein kinase C zeta. *J Leukoc Biol* 2003, **73**, 802-814.
16. Wang JP: Characterization of maleimide-activated Ca²⁺ entry in neutrophils. *Biochem Pharmacol* 2003, **65**, 1923-1929.
17. Dewitt S, Hallett MB: Cytosolic free Ca²⁺ changes and calpain activation are required for α 5 β 1 integrin-accelerated phagocytosis by human neutrophils. *J Cell Biol* 2002, **159**, 181-189.
18. Tintinger G, Steel HC, Anderson R: Taming the neutrophil: calcium clearance and influx mechanisms as novel targets for pharmacological control. *Clin Exp Immunol* 2005, **141**, 191-200.
19. Sarantis H, Gray-Owen SD: The specific innate immune receptor CEACAM3 triggers neutrophil bactericidal activities via a Syk kinase-dependent pathway. *Cell Microbiol* 2007, **9**, 2167-2180.
20. Fudala R, Krupa A, Matthay MA, Allen TC, Kurdowska AK: Anti-IL-8 autoantibody: IL-8 immune complexes suppress spontaneous apoptosis of neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2007, **293**, L364-374.
21. Pearn L, Fisher J, Burnett AK, Darley RL: The role of PKC and PDK1 in monocyte lineage specification by Ras. *Blood* 2007, **109**, 4461-4469.
22. Ibrahim S, Calzada C, Pruneta-Deloche V, Lagarde M, Ponsin G: The transfer of VLDL-associated phospholipids to activated platelets depends upon cytosolic phospholipase A2 activity. *J Lipid Res* 2007, **48**, 1533-1538.