EFFECT OF THE PURINE DERIVATIVE MYOSEVERIN AND OF ITS ANALOGUES ON CULTURED HYBRIDOMA CELLS

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Two 2,6,9-trisubstituted purine derivatives, 9-isopropyl-2,6-bis[(4-methoxybenzyl)amino]-9*H*-purine (myoseverin, PMYO, **1**) and 9-isopropyl-2,6-bis[(2-methoxybenzyl)amino]-9*H*-purine (OMYO, **2**), and two 6,9-disubstituted derivatives, 9-isopropyl-6-[(4-methoxybenzyl)amino]-9*H*-purine (**3**) and 9-isopropyl-6-[(2-methoxybenzyl)amino]-9*H*-purine (**4**), were synthesized with the aim to examine their cell proliferation inhibiting activity, and possible additional effects in cultures of hybridoma cells producing monoclonal antibody. The substances were tested over a concentration range from 0.003 to 30 μmol l^{-1}. The most active compound **1** caused a total loss of cell viability at 1 μmol l^{-1}, while its isomer **2** showed the same effect at 10 μmol l^{-1} concentration. In the presence of compound **1**, but not of compound **2**, the character of the cell cycle phases profile changed dramatically, most cells being arrested in the G_2/M phase. At intermediate concentrations of compound **2** a substantially higher viable cell concentration was observed, relative to control. These differences demonstrated the principal significance of the position of the methoxy groups on the benzene rings for the biological effect. The 6,9-disubstituted derivatives **3** and **4** were without significant effect in the whole range of concentrations tested. The enhancement of monoclonal antibody production, observed in certain concentration intervals of added substances, was of marginal character.

Keywords: Purines; CDK Inhibitors; Cytokinins; Cytostatics; Hybridoma cells; Monoclonal antibodies.

Achievement of efficient regulation of cell proliferation by synthetic low-molecular-weight substances is desirable not only in the treatment of tumours, but also in biological technologies employing transformed animal cell lines cultured *in vitro*. While in oncology the cancerostatic agent is expected to arrest tumour cell growth and finally to induce programmed death of tumour cells, in biotechnology arresting of cell growth must not be accompanied by an increased rate of cell death. Therefore, the action of new cancerostatics is first explored in laboratory assays in order to select substances having the capacity of arresting or slowing down cell proliferation when applied at substantially lower concentrations than those inducing programmed cell death.

Suppression of cell growth is particularly advantageous in the production of monoclonal antibodies by hybridoma cells, because slow growth is evidently accompanied by an increased rate of antibody production[1]. Following this line of research we demonstrated a certain enhancement of monoclonal antibody yield with an acyclic nucleotide analogue[2], and with the 2,6,9-trisubstituted purine derivative bohemine[3]. Bohemine belongs to analogues of aromatic cytokinins, *i.e.*, plant hormones, similarly to olomoucine or roscovitine[4]. The main mechanism of action of these substances has been recognized as the competition with ATP binding to cyclin-dependent kinases[5]. Libraries of 2,6,9-trisubstituted purines were prepared and numerous inhibitors of cell proliferation were identified. It was concluded that purine derivatives affect distinct metabolic and regulatory pathways mediating different cellular functions[6]. A similar conclusion was reached with bohemine action when employing proteomics technology[7], or kinetic analysis of bohemine-treated hybridoma cultures[3].

Another 2,6,9-trisubstituted purine, myoseverin, was found to induce extensive alterations of the level of gene expression and myoblast–myotube conversion[8]. Although this microtubule-binding substance does not inhibit cyclin-dependent kinases, its interaction with microtubules, which are generally asssociated with mitosis and the process of cell division, classified this compound in the group of possible regulators of cell growth. The aim of the present study was to examine cellular effects of myoseverin and a few analogues on a hybridoma cell line, and to investigate possible influence of these compounds on the rate of production of monoclonal antibody.

EXPERIMENTAL

Chemicals

6-Chloropurine and 2,6-dichloropurine were obtained from Aldrich. Cell culture media and supplements were from Life Technologies. Reagents for the synthesis of purine derivatives, dimethylsulfoxide for stock solution of the derivatives, and chemicals used for the assays were of analytical grade. DNA Prep Reagent kit was from Coulter Immunology (Hialeah (FL), U.S.A.).

Chromatography

Thin-layer chromatography (TLC) was carried out using aluminium sheets with silica gel F_{254} from Merck. Spots were visualized under UV light (254 nm). Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography.

Instruments and Analyses

Melting points were determined on a Kofler block and are uncorrected. ^{1}H NMR spectra (δ, ppm; *J*, Hz) were measured on Varian INOVA 400 (400 MHz) or on Varian Gemini 300 (300 MHz) spectrometers. ^{13}C NMR spectra were measured on Varian INOVA 400 (100 MHz) spectrometer. All spectra were obtained at 25 °C using tetramethylsilane as internal standard. Mass spectra were measured on a MS Waters/Micromass (ZMD-detector, direct inlet, ESI, + ions). Elemental analyses were performed by Central Laboratory, Institute of Chemical Technology, Prague.

Purine Derivatives

6,9-Disubstituted and 2,6,9-trisubstituted purine derivatives were prepared according published procedures[9–14] from commercially available 6-chloropurine or 2,6-dichloropurine.

9-Isopropyl-2,6-bis[(4-methoxybenzyl)amino]-9H-purine (myoseverin, PMYO, **1**)[6a,8,14]. White crystals, m.p. 108–111 °C, crystallized from ether.

9-Isopropyl-2,6-bis[(2-methoxybenzyl)amino]-9H-purine (OMYO, **2**). Yield 55%; yellowish crystals, m.p. 74–84 °C, crystallized from ether. MS (CV 20 V), *m/z* (rel.%): 433 (100) $[M + H]^+$, 434.3 (25). ^{1}H NMR (400 MHz, $CDCl_3$): 1.523 (d, 6 H, *J* = 6.8, $(CH_3)_2CH$); 3.865 (s, 3 H, CH_3O); 3.877 (s, 3 H, CH_3O); 4.647 (hept, 1 H, *J* = 6.8, $(CH_3)_2CH$); 4.663 (d, 2 H, CH_2); 4.800 (bd, 2 H, *J* = 4.4, CH_2); 5.227 (t, 1 H, *J* = 6.3, NH); 5.917 (bs, 1 H, NH); 6.824–6.890 (m, 4 H, H-arom); 7.190–7.25 (m, 2 H, H-arom); 7.469 (s, 1 H, H-8). Proton 2D-COSY, TOCSY and HMQC experiments were used for the assignment of signals.^{13}C NMR (100 MHz, $CDCl_3$): 22.582, 39.864, 41.333, 46.061, 55.290, 55.317, 110.116, 110.156, 120.313, 120.433, 127.986, 128.407, 128.507, 129.508, 129.669, 134.103, 154.945, 157.669, 159.586. Some signals of quarternary carbons were not observed. For $C_{20}H_{28}N_6O_2$ (432.5) calculated: 66.65% C, 6.53% H, 19.43 % N; found: 66.60% C, 6.60% H, 19.37% N.

9-Isopropyl-6-[(4-methoxybenzyl)amino]-9H-purine (**3**). Yield 70%; white crystals, m.p. 96– 98 °C, crystallized from chloroform–ether. MS (CV 25 V), *m/z* (rel.%): 298 (100) $[M + H]^+$, 299.3 (18). ^{1}H NMR (300 MHz, $CDCl_3$): 1.61 (d, 6 H, *J* = 6.6, $(CH_3)_2CH$); 3.79 (s, 3 H, CH_3O); 4.85 (hept, 1 H, *J* = 6.6, $CH(CH_3)_2$); 4.85 (bs, 2 H, CH_2NH); 6.87 (d, 2 H, *J* = 8.2, H-arom); 7. 33

(d, 2 H, *J* = 8.2, H-arom); 7.82 (s, 1 H, H-8); 8.39 (s, 1 H, H-2). For $C_{16}H_{19}N_5O$ (297.4) calculated: 64.63% C, 6.44% H, 23.55% N; found: 64.53% C, 6.50% H, 23.56% N.

9-Isopropyl-6-[(2-methoxybenzyl)amino]-9H-purine (**4**). Yield 72%; white crystals, m.p. 119–122 °C, crystallized from ether. MS (CV 20 V), *m/z* (rel.%): 298 (100) $[M + H]^+$, 299 (17). ^{1}H NMR (300 MHz, $CDCl_3$): 1.59 (d, 6 H, *J* = 6.6, $(CH_3)_2CH$); 3.90 (s, 3 H, CH_3O); 4.84 (hept, 1 H, *J* = 6.6, $CH(CH_3)_2$); 4.86 (bs, 2 H, CH_2NH); 6.30 (bs, 1 H, NH); 6.86–6.94 (m, 2 H, H-arom); 7.23–7.29 (m, 1 H, H-arom); 7.38–7.44 (m, 1 H, H-arom); 7.89 (s, 1 H, H-8); 8.40 (s, 1 H, H-2). For $C_{16}H_{19}N_5O$ (297.4) calculated: 64.63% C, 6.44 % H, 23.55 % N; found: 64.53% C, 6.61 % H, 23.28 % N.

Cell Culture Assays

Mouse hybridoma ME-750 was cultured in DMEM/F12/RPMI 1640 (2 : 1 : 1) medium supplemented with amino acids and with the iron-rich protein-free growth-promoting mixture as described in detail before[3,15]. The cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

The tested substances were dissolved in dimethylsulfoxide to 10 mmol l^{-1} concentration. Further dilutions were prepared in the culture medium.

Assays of the action of the tested substances were set up in a total volume of 6.0 ml in 25 cm^2 T-flasks. The initial cell concentration was $(250 \pm 50) \cdot 10^3$ cells ml^{-1}. Standard duration of the assays was 6 days. Viable cells and dead cells were counted in a hemocytometer using Trypan Blue exclusion test. Assays of the activity of various concentrations of the tested substances were repeated in at least three independent experiments. The experimental error involved in the estimation of cell concentration was ±10%. The concentration of monoclonal antibody (MAb) produced by the cells in the culture medium was determined by immunoturbidimetry in three parallels for each sample[16]. The experimental error associated with the estimation of the MAb concentration was ±10%.

Distribution of Cell Cycle Phases

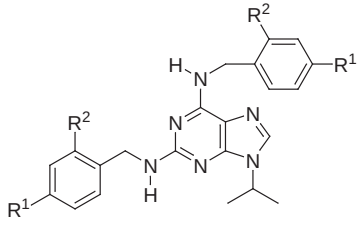
For determination of cell kinetics the cells ($5–10 \cdot 10^6$) were permeabilized and stained using DNA Prep Kit. After 30-min incubation, the samples were measured with a FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes (NJ), U.S.A.) and the data were analyzed by ModFit LT 3.1 DNA analysis software (Verity Software House, Inc., Topsham (ME), U.S.A.). Percentages of cells in G_0/G_1, S and G_2/M phases were evaluated. Samples from duplicate flasks for each sample were analysed and averaged.

RESULTS

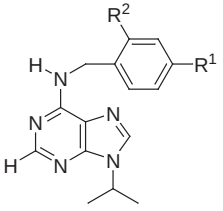
The biological activity of the purine derivatives **1**–**4** was tested on the model of a mouse hybridoma producing monoclonal antibody of the IgG class. The growth curves of the hybridoma cultures were analogous to those described in the preceding paper reporting on the activity of the purine derivative bohemine[3]. Similarly to the findings of our previous work, the most pronounced differences between the parameters of the control culture and those of the cultures supplemented with the tested compounds could

be observed at the beginning of the decline phase of the culture. Therefore, the culture parameters determined on day 6 were used for the evaluation of the activity of tested substances.

The effect of the four substituted purines was tested over a concentration range spanning several orders of magnitude. The trisubstituted derivative PMYO (myoseverin, **1**) was found to cause a sharp decrease in the number of viable cells in the concentration interval between 0.1 and 1 μmol l^{-1}. A similar sharp decrease was observed with OMYO (**2**) between 1 and 10 μmol l^{-1}. However, the effect of OMYO (**2**) was more complex. At 0.3 and 1 μmol l^{-1} concentrations OMYO (**2**) significantly stimulated cell growth (Table I).



1, 2



3, 4

	R^1	R^2
1	OMe	H
2	H	OMe
3	OMe	H
4	H	OMe

Slight but significant increase of the MAb yield could be observed upon supplementation of the culture with PMYO (**1**) at concentrations from 0.01 to 0.1 μmol l^{-1}. The increase in the MAb yield caused by OMYO (**2**) was less convincing. The values of the viable cells/total cells ratio were in the range of standard variations with most concentrations of both derivatives. This ratio dropped substantially in parallel with the drop of viable cells at higher concentrations of the compounds. The data in Table I showed a seemingly striking fact that the relative MAb values found in the range of suppressed growth were higher than the corresponding relative viable cell concentrations. This finding is quite conceivable, if we realize that the cells do not stop their metabolism and do not die immediately under the influence of the agents, but live for a certain time period, under limited proliferation, and synthesize and secrete antibody molecules. We can conclude, from all the above data, that the position of the methoxy group in the benzene ring, *para* or *ortho*, has a high impact on the nature of the biological effect of trisubstituted purine derivatives.

The assays with disubstituted purines **3** and **4** yielded a picture rather different from the results of the above reported assays with trisubstituted derivatives. Even though the range of concentrations of disubstituted derivatives was extended to 30 μmol l^{-1}, no pronounced effect on cell growth or on MAb production could be demonstrated (Table II). Only compound **3** at 0.1 and 1 μmol l^{-1} concentrations yielded viable cells and MAb values slightly exceeding the interval of experimental errors. Thus, the bulky substituent at position 2 of the purine skeleton is obviously essential for the effect of purine derivatives on cell proliferation.

The effect of PMYO (**1**) and OMYO (**2**) on the distribution of cell cycle phases in growing hybridoma cultures was examined by flow cytometry. Cell populations after 3 days of culturing in the presence of the agents were subjected to analysis. The concentrations of the agents were selected, on the basis of the data of Table I, to achieve significant suppression of cell growth on the one hand, and to avoid induction of apoptosis on the other

TABLE I
Effect of 2,6,9-trisubstituted purine derivatives PMYO (**1**) and OMYO (**2**) on growth and production of hybridoma cells[a]

Concentration μmol l^{-1}	PMYO (**1**)			OMYO (**2**)		
	viable cells[b]	MAb	viable cells/ total cells	viable cells	MAb	viable cells/ total cells
	% of control			% of control		
0 (control)	100	100	0.58	100	100	0.58
0.003	110	112	0.63	101	98	0.60
0.01	108	122	0.65	93	104	0.62
0.03	110	125	0.64	99	115	0.64
0.1	101	128	0.63	95	111	0.58
0.3	4	64	0.07	137	112	0.52
1	0	31	0	190	107	0.59
3	–	0	–	49	63	0.22
10	–	–	–	0	10	0

[a] Parameters of the cultures determined after 6 days of culture duration are given. [b] Parameters of control cultures (mean ± SD): Viable cells (10^3 cells ml^{-1}) 1 220 ± 90; MAb (mg l^{-1}) 48 ± 6.

hand. The effect of OMYO (**2**) was found insignificant in comparison with the control culture (Table III). However, the other isomer PMYO (**1**) provoked a dramatic change in cell cycle phase distribution. Most cells accumulated in the G_2/M phase. In addition, cells with higher than tetraploid content were observed. The flow cytometric analysis thus confirmed the

TABLE II
Effect of 6,9-disubstituted purine derivatives **3** and **4** on growth and production of hybridoma cells[a]

Concentration μmol l^{-1}	Compound (**3**)			Compound (**4**)		
	viable cells[b]	MAb	viable cells/ total cells	viable cells	MAb	viable cells/ total cells
	% of control			% of control		
0 (control)	100	100	0.59	100	100	0.59
0.01	107	110	0.68	106	110	0.60
0.1	103	112	0.65	105	106	0.62
1	114	120	0.67	99	100	0.65
10	107	102	0.65	102	98	0.60
30	101	108	0.64	94	109	0.62

[a] Parameters of the cultures determined after 6 days of culture duration are given. [b] Parameters of control cultures (mean ± SD): Viable cells (10^3 cells ml^{-1}) 1 260 ±70; MAb (mg l^{-1}) 52 ± 5.

TABLE III
Distribution of cell cycle phases in cultures supplemented with PMYO (**1**) and OMYO (**2**)

Added substance	Cell cycle phase[a], %		
	G_0/G_1	S	G_2/M
None (control)	65.6 ± 2.6	34.3 ± 2.7	0.2 ± 0.4
PMYO (**1**), 0.3 μmol l^{-1}	9.0 ± 1.3	10.9 ± 0.5	80.2 ± 3.8[b]
OMYO (**2**), 0.3 μmol l^{-1}	68.6 ± 1.7	31.8 ± 1.6	0.1 ± 0.2

[a] Mean ± SD. [b] Some cells contained >4 times more DNA than the cells in the G_0/G_1 phase.

significance of the position of methoxy groups in the benzene ring for the biological activity.

DISCUSSION

Our previous efforts at enhancing the production of MAbs through manipulation with the cell cycle of the producing hybridoma cells made use of nucleoside-derived agents that were able to control the rate of DNA synthesis[2], or purine-derived agents that inhibited cyclin-dependent kinases[3]. Microtubules represent another of the central elements of the process of mitosis. After the activity of the purine derivative myoseverin on microtubules was revealed[8], we decided to explore the possibility of cell cycle manipulation with this substance and with several of its analogues.

The characteristic feature of the action of PMYO (myoseverin, **1**) and OMYO (**2**) was the steep course of the dependence of cell survival on the concentration of the agents. Another striking finding was the action of OMYO (**2**) at intermediate concentrations resulting in a substantially higher viable cell concentration relative to control (Table I). The effects of trisubstituted purine derivatives are complex and cannot be evidently interpreted as a simple dose-dependent cytostatic activity. Support for this conclusion can also be found in previous analyses of other authors dealing with the action of myoseverin on myotubes[8]. Myoseverin increased the expression levels of genes involved in purine catabolism and detoxification. On the other hand, myoseverin down-regulated the expression of some apoptosis-inducing genes.

Profound changes in the rate of cell proliferation and the rate of cell death were expected to be accompanied by changes in the profile of the cell cycle phases. This aspect was analysed in cell populations that were exposed to concentrations of compounds causing growth suppression but not an immediate cell death. The flow cytometer analyses demonstrated a dramatic difference between the mild action of OMYO (**2**) and the collapse of cell cycle progression following the addition of PMYO (**1**). Hybridoma cells are hypotetraploid, due to their somatic-hybrid nature. Obstacles arising in the accomplishment of the mitotic process, such as the assumed interaction of PMYO (**1**) with microtubules, will evidently result in formation of cells possessing a higher than tetraploid, possibly hypooctaploid, DNA content (Table III).

The application of the purine derivatives under investigation did not lead to any pronounced enhancement of the MAb yield. No correlation was found between the enhancement of viable cell concentration and the final

MAb concentration, except for the marginal increase of both parameters at 1 μmol l^{-1} compound **3** (Tables I and II). Slightly elevated values of MAb were observed at intermediate concentrations of PMYO (**1**) (0.01 to 0.1 μmol l^{-1}). The viable cell concentration, however, remained at the level of that in the control, within the limits of error. In OMYO (**2**) supplemented cultures the substantial increase in the viable cell concentration (at 0.3 μmol l^{-1}) was not accompanied by a corresponding increase in the MAb yield. It follows, that the determinants controlling the production rate of MAb are obviously different from those controlling cell growth and viability.

Although the present study does not result in a straightforward instruction how to enhance MAb yields through addition of purine derivatives, it extends the knowledge in the field of interactions of various purine derivatives with cells, and contributes to a basis for targeted manipulations with animal cell systems *in vitro*.

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