

Received 23 July 2000

Accepted 14 November 2000

**EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF
FOUR COMPOUNDS EXERTING ANTIMUTAGENIC EFFECTS ON
HUMAN LYMPHOCYTES IN VITRO**

KAZIMIERZ GASIOROWSKI*, BARBARA BROKOS
and HELENA TABAKA

Wroc³aw Medical University, Department of Basic Medical Sciences,
14 Kochanowskiego Str., 51-601 Wroclaw, Poland.

Abstract: Four compounds previously described as antimutagenic for human lymphocytes *in vitro* were tested on their immunomodulatory activity in lymphocyte cultures. The standard immunocytochemical methods were applied for microscopic examination of the percentual representation of the main lymphocyte subpopulation. The results imply that all of the tested compounds exhibited significant immunomodulatory effect, with that of fluphenazine being the strongest, whereas that of todralazine is the weakest. Two of the tested compounds: anthocyanins from *Aronia melanocarpa* fruit, and alkylresorcinols from cereal grains, also exhibited a distinct immunomodulatory activity, and it deserves adequate attention as an activity exerted by natural products, commonly present in regular human diet.

The analysis of the proliferating cell fraction, and the estimation of the cell proliferation rate suggest that the effect of the tested compounds might depend on an increase in the number of lymphocytes which expressed their differentiation antigens on the cell membranes.

Key Words: Antimutagens, Immunomodulatory Effect, Lymphocyte Subpopulation Frequencies, Proliferation Rate.

INTRODUCTION

The antimutagenic action of four compounds was analysed and described in a battery of standard short-term tests. Two of the compounds were natural, isolated from plants (anthocyanins from the fruit of *Aronia melanocarpa* and alkylresorcinols from cereal grains), whereas two others were synthetic, currently-used drugs (todralazine – an antihypertensive drug from the hydralazinophthalazine family, and fluphenazine – a psychotropic drug, a member of the phenothiazine group). A series of papers we documented the strong antimutagenic effect of both anthocyanins (AN) [1, 2] and alkylresorcinols (AR) [2, 3], and todralazine (TDR) [4-6] and fluphenazine (FPh) [6, 7]. Since most of the results were obtained in standard cytogenetic tests with human lymphocyte cultures, it was important to examine the influence of the tested compounds on the proliferation rate and the frequencies of the main lymphocyte subpopulations *in vitro*. The antimutagenic activity of the compounds was evaluated in lymphocytes obtained from heavy smoking blood donors. This group of people clearly requires the development of an effective antimutagenic strategy, because heavy smokers are exposed to high mutation risk. An examination of the immunomodulatory influence of the tested compounds on lymphocytes obtained from smoking blood donors appeared to be an important complementation to the research cited above.

The aim of this study is to use immunocytochemical methods to evaluate the impact of four antimutagenic compounds on human lymphocyte cultures.

MATERIALS AND METHODS

Chemicals

The blood-cell separation solution – Histopaque-1077 – and components of cell culture media: RPMI 1640, foetal calf serum (FCS) and L-glutamine were obtained from Sigma (St.Louis, USA). Phytohemagglutinin (PHA-M) was obtained from Gibco (Gaithersburg, USA). 5²-bromodeoxyuridine (BrdUrd), demecolcine, dimethyl sulfoxide (DMSO) and the stains: azure II, eosin B and trypan blue were obtained from Sigma (St.Louis, USA). The other reagents used for buffers and culture media preparation were from POCH (Gliwice, Poland).

Antibodies

The monoclonal antibodies used for the detection of lymphocyte surface antigens were purchased from DAKO A/S (Glostrup, Denmark). The following mouse monoclonal antibodies were applied: anti-CD4 (clone MT310), anti-CD8 (clone DK25), anti-CD22 (pan-B, clone To15), anti-CD56 (NK-cells, clone T199), and anti-Ki-67 antigen (clone Ki-67). An LSAB Kit, also from DAKO A/S (Glostrup, Denmark), was applied to reveal the antibody-bound cells by

means of the peroxidase-anti-peroxidase (PAP) system with diaminobenzidine (DAB)+H₂O₂ as a chromogen.

The tested compounds

Alkylresorcinols (5-n-alk(en)ylresorcinols) were isolated and purified from a rye bran milling fraction at the Department of Lipids and Liposomes, Wrocław University, following the procedure described previously [10, 11]. The molecular weight of AR from such a preparation was established as being close to 376.33. The lyophilized powder was stored in a freezer.

Anthocyanins (AN) were isolated from the fruit of *Aronia melanocarpa*, extracted and purified at the Department of Fruit and Vegetable Technology, Wrocław Agricultural University, following the procedure included in Polish Patent No. PL 158707 and described in previous papers [8, 9]. The molecular weight of the AN was assumed as closely approximating 449.40. The lyophilized AN powder was stored in a freezer.

Todralazine hydrochloride powder (TDR), research grade, molecular weight 268.7, was kindly supplied by Polfa (Pabianice, Poland), and fluphenazine dihydrochloride powder (FPh), research grade, molecular weight of 510.4, was provided by Jelfa (Jelenia Góra, Poland). Both powders were stored at room temperature.

On the day of the experiment, the tested compounds were dissolved with bidistilled water (AN, TDR, FPh) or with DMSO (AR), and filtered through a 0.2 µm Milipore filter (Sartorius, Germany). Serial dilutions were prepared and the compounds' solutions were added to the cell culture medium at a volume of 50 µl, at the dose needed to obtain the presumed final concentration. The pH value of the culture medium containing the tested compounds was checked with a MP125 pH meter equipped with an Inlab 413 electrode (Mettler Toledo, Greifensee, Switzerland)

Blood cell separation

Heparinized blood was obtained by venipuncture from three healthy male volunteers aged 25-30, each smoking 20-30 cigarettes per day. The cells were separated by a single-step density gradient centrifugation technique after layering the heparinized blood upon the Histopaque-1077 layer [12]. Isolated lymphocytes were washed in phosphate buffered saline (PBS, pH 7.2), counted under a microscope and kept for biological tests.

Lymphocyte cultures

The viability of the lymphocytes was evaluated with the standard trypan blue-exclusion test and examined under a microscope after 18h culture in the presence of the compounds (CO₂-incubator, 37°C). In this test, the maximal concentrations of the tested compounds for which an increase in the number of

dead cells was not yet noticeable were established at: 60 µg/ml for AN, 40 µg/ml for AR, 80 µg/ml for TDR and 5 µg/ml for FPh.

Lymphocytes were cultured in 24-well plastic dishes at a density of 5×10^5 cells/ml in the complete medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, 25 µg/ml gentamicin), and the cultures were stimulated to mitogenesis with PHA-M (1% v/v). The tested compounds were added at a volume of 50 µl, each to a separate series of cultures, and were present for the whole culture time, i.e. for 72h. The control cultures contained a solvent added at a volume of 50 µl, instead of an antimutagen. The final volume of each culture was 2.65 ml.

Cytogenetic estimation of proliferation rate

Proliferating cells were labelled with the thymidine analogue BrdUrd (35 µM) added to the culture for the last 48h. The mitotic spindle poison (demecolcine, 10 µg/ml) was added for the last 3 hours of the culture time. The cultures were harvested following the standard cytogenetic method. Cell smears prepared on glass slides were air-dried for 3 days. The differential chromatid staining was revealed by immersing the slides in a 2xSSC solution (0.03M sodium citrate in a 0.3M sodium chloride-aqueous solution) at 62°C for 30 min. and simultaneously illuminating them under a UV lamp [13]. Then the slides were stained with a mixture of dyes: azure II/eosin B [14]. The replication indices of the lymphocyte cultures were estimated by counting the number of metaphases in the first (M_1), second (M_2) and third (M_3) division in the presence of BrdUrd. Two slides were analysed under a microscope for each experimental point, and 300 metaphases were counted on each slide. Finally the replication indices were calculated according to the formula: $RI = (M_1 + 2M_2 + 3M_3)/100$.

Immunocytochemical staining

After 72h culture in the presence of the tested compounds the cultures were spun out and washed with PBS, and glass slide smears were made. The slides were dried overnight, and then fixed with a mixture containing methanol, acetone and formaline (19:19:2 v/v) for 90 sec. Afterwards, the slides were immersed for 30 min. with a solution of the specific monoclonal antibodies, able to bind to the tested lymphocyte surface antigen. The cells which bound the specific antibody were visualised with a DAKO LSAB Kit for PAP staining [15, 16]. The frequencies of stained cell subpopulations were counted under a microscope. For each experimental point two replicate slides were analysed and 3000 cells were counted on each slide. The percentage of positively stained cells was estimated and compared to the percentage of cells stained in the control culture (without the tested compounds) and expressed as the experimental versus control (E/E_0) ratio.

Statistical analysis

The concentration of each tested compound was expressed as $\log [x+1]$; x = concentration of the compound in the culture medium, and the obtained results were given as E/E_0 quotients. The dose-response relations were estimated by calculation of regression equations following standard statistical methods [e.g. 17].

RESULTS AND DISCUSSION

The immunomodulatory effect of the tested compounds was estimated in 72h cultures of cells in the standard lymphocyte culture medium (RPMI 1640, 10% FCS, 2mM L-glutamine, gentamicin 25 $\mu\text{g/ml}$) containing a lectin (PHA-M, 1% v/v). It was established that in the range of the tested concentrations the compounds did not significantly change the pH value of the culture medium, although a mild acidification effect was noted in the case of TDR at a concentration of 100 $\mu\text{g/ml}$ (the pH level decreased by 0.03). Therefore lower concentrations of TDR were chosen (up to 80 $\mu\text{g/ml}$), in which changes of the pH value were not observed.

The impact of the tested compounds on the frequencies of the main lymphocyte subpopulations (CD4, CD8, B, and NK) as well as on lymphocyte proliferation (Ki-67 antigen expression, replication index - RI) was estimated, and regression equations were calculated to analyse the dependence of the observed changes in lymphocyte numbers on the concentration of the tested compounds. In the case of each tested compound, some of the regression lines slopes were not significant. They were not included in the figures and corresponding tables, as their results did not depend on the concentration of the tested compound.

The influence of AN and AR on the percentual representation of the main lymphocyte subpopulations and also on lymphocyte proliferation are presented in Fig.1 in the form of regression lines describing dose-response relations. The regression equations are given below in Tab.1.

As may be seen in Fig.1 (top), the impact of AN on the main lymphocyte subpopulations was especially strong in the case of the NK cell frequency. At the highest tested concentration of AN (60 $\mu\text{g/ml}$), the percentual representation of the NK cells was 1.4 times as high as in the control cultures (without AN). The proportion of CD4/CD8 lymphocytes increased mildly, and at the highest concentration of AN the proportion was about 15% higher than in the control cultures. Lymphocyte B-frequencies did not significantly vary in the range of AN concentrations (data not included in Fig.1). The impact of AN on lymphocyte PHA-induced proliferation caused a marked increase of Ki-67-antigen-expressing cells (1.8-fold as compared to the control culture), and a mild (6%), but significant increase of the replication index (RI).

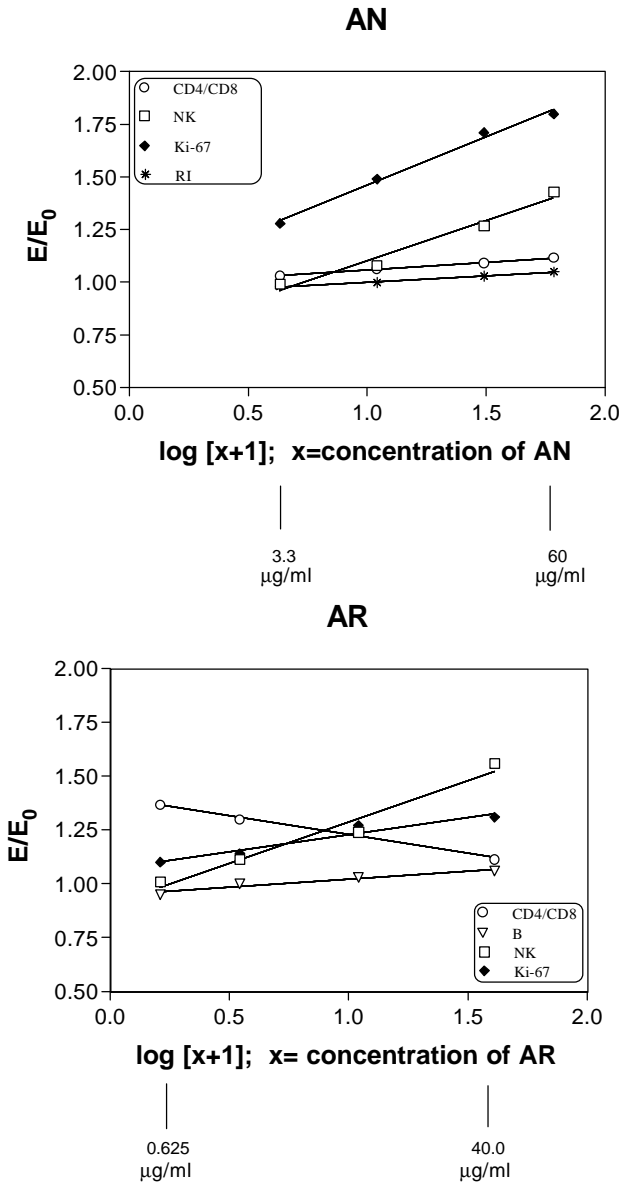


Fig.1. The impact of anthocyanins (AN) and alkyresorcinsols (AR) on the percental representation of the main lymphocyte subpopulations and lymphocyte PHA-induced proliferation *in vitro*. The results obtained in the presence of AN or AR (E) were compared to the relevant control culture (E₀), i.e. lymphocytes cultured without the tested compounds. The dose-dependent effects were calculated with regression equations.

Tab.1. Regression analysis of the data given in Fig.1.

Regression equation: $a_0 + a_1 \log [x+1]$							
	Lymphocytes:	a_0	SE	a_1	SE	r	P
AN	CD4/CD8	0.985	0.0043	0.072	0.0032	0.99	0.0021
	NK	0.718	0.0616	0.383	0.0469	0.98	0.0147
	Ki-67+	1.001	0.0378	0.459	0.0288	0.99	0.0039
	RI	0.939	0.0041	0.061	0.0032	0.99	0.0026
AR	CD4/CD8	1.404	0.0148	-0.175	0.0147	0.99	0.0155
	B	0.950	0.0134	0.074	0.0133	0.97	0.0305
	NK	0.904	0.0521	0.383	0.0521	0.98	0.0179
	Ki-67+	1.002	0.0494	0.212	0.0492	0.95	0.0498

In Fig.1 (bottom) the effect of AR on the percentual representation of the main lymphocyte subpopulations and on PHA-induced proliferation are given. It can be seen that the percentual representation of the NK cells increased markedly in the presence of AR, by 60% at the highest tested AR concentration (40 $\mu\text{g/ml}$). In the case of the CD4/CD8 ratio, an increase of 40% was observed at the lowest tested AR concentration (0.625 $\mu\text{g/ml}$), whereas at the highest concentration (40 $\mu\text{g/ml}$) the ratio was 10% higher than in the control culture. The slope of the regression line describing this relation exhibited a negative direction, though it should be stressed that in the range of the tested concentrations all results were higher than in the control cultures. The frequency of the B cells increased mildly and at the highest tested AR concentration an increase of about 10% was estimated. The rate of lymphocyte replication (RI) did not alter significantly in the presence of AR (data not shown). On the other hand, AR increased 1.3-fold the representation of Ki-67 expressing cells, which could reflect its ability to recruit cells to the proliferation cycle.

The impact of TDR and FPh on lymphocyte subpopulations and on PHA-induced proliferation is shown in Fig.2 and the corresponding Tab.2. As may be seen in Fig.2 (top), the regression lines describing the impact of TDR exhibited negative directions, although all the results were higher than in the related controls. The CD4/CD8 ratio was highest at lower concentrations of TDR (e.g. 1.45-fold at 5 $\mu\text{g/ml}$), whereas at the highest concentration it was approximately at the same level as in the control cultures. The B-cell frequency was 75% higher and 35% higher in the lowest and in the highest concentrations, respectively. The effect of TDR on the NK cell percentual representation did not depend on the drug concentration, therefore it was not included in Fig.2.

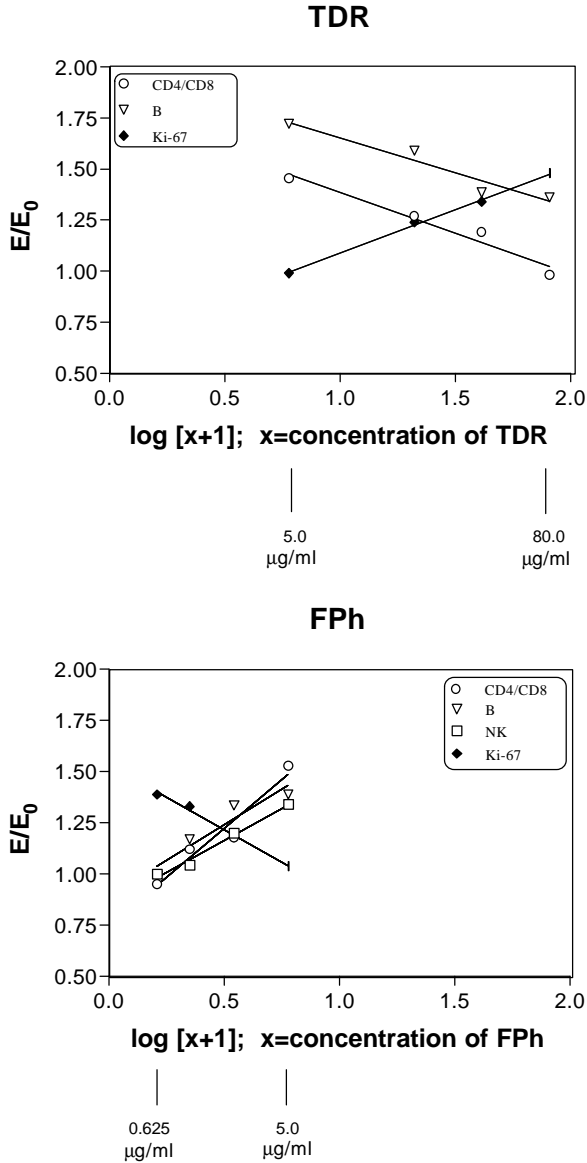


Fig.2. The influence of todralazine (TDR) and fluphenazine (FPh) on the percental representation of the main lymphocyte subpopulations and lymphocyte PHA-induced proliferation *in vitro*. The results were compared to the relevant controls (without the tested compounds), and expressed as an E/E_0 ratio. The dose-response relations were calculated with regression equations.

The comparison of the replication indices proved that the impact of TDR on lymphocyte PHA-induced proliferation was not significantly dose-dependent in the case of the replication rate (RI). In the case of Ki-67 antigen-bearing cells the increase was concentration-dependent, and at the highest concentration an increase of even about 50% was noticed.

The impact of FPh on the lymphocyte subpopulations and lymphocyte proliferation was given in Fig.2 (bottom) and related Tab.2. As may be seen in Fig 2 (bottom), the regression lines describing the effect of FPh on the percentual representation of the NK cells, the CD4/CD8 ratio and the B-cells exhibited very similar slopes. The results at the highest concentration of FPh (5 µg/ml) were 1.35 to 1.5times as high as in the controls.

FPh did not influence the replication rate (RI) of the lymphocytes. At lower concentrations of the drug (0.625-2.5 µg/ml), an increased percentage of Ki-67+ cells was recorded, (even 1.4-fold), whereas at the highest concentration (5 µg/ml), there was a mild decrease in comparison to the control cultures. It indicates, that lower concentrations of FPh could increase the recruitment of lymphocytes to the generation cycle, but higher concentrations could increase the numbers of quiescent, non-proliferating cells.

Tab.2. Regression analysis of the data given in Fig.2.

Regression equation: $a_0 + a_1 \log [x+1]$

	lymphocytes:	a_0	SE	a_1	SE	r	P
TDR	CD4/CD8	1.785	0.0860	-0.399	0.0587	0.98	0.0209
	B	1.997	0.0964	-0.344	0.0657	0.96	0.0346
	Ki-67+	0.625	0.0222	0.428	0.0151	0.99	0.0012
FPh	CD4/CD8	0.742	0.0876	0.961	0.1694	0.97	0.0297
	B	0.893	0.0836	0.696	0.1617	0.95	0.0498
	NK	0.851	0.0280	0.626	0.0541	0.99	0.0074
	Ki-67+	1.625	0.0203	-0.637	0.0392	0.99	0.0038

Since the tested compounds differed noticeably in their cytotoxicity, the ranges of their concentrations applied to lymphocyte cultures differed significantly. Therefore, the recalculation of the data presented in the figures and tables in relation to the concentration and the molecular weight of each compound was necessary for a direct comparison of their immunomodulatory action. The results of these calculations are collected and presented in Tab.3.

As may be seen in Tab.3, FPh exhibited a decidedly stronger immunomodulatory activity than the three other compounds, considering the concentration and the molecular weight of the compounds. It should be stressed that all the results of FPh impact on human lymphocyte subpopulations were

above the level of the control culture (without FPh). The data suggest a strong immunostimulatory action of FPh. AN and AR also exhibited an immunostimulatory activity, although significantly smaller than that of FPh. Among the tested compounds, TDR exhibited the weakest action on lymphocyte cultures.

Tab.3. The immunomodulatory activity of the four tested compounds calculated per 1mM of the compound present in the culture medium (mean \pm SD, n=3).

AN - anthocyanins from *Aronia melanocarpa*, AR - alkylresorcinols from cereal grains, FPh - fluphenazine, TDR – todralazine.

<i>lymphocytes</i>	<i>tested compounds</i>			
	AN	AR	FPh	TDR
CD4/CD8	0.70 \pm 0.056	2.43 \pm 0.122*	67.66 \pm 8.119	1.70 \pm 0.070*
B	NS	2.01 \pm 0.181	41.50 \pm 5.395	1.29 \pm 0.228*
NK	3.49 \pm 0.244	5.26 \pm 0.579	35.00 \pm 3.150	NS
Ki-67+	4.12 \pm 0.371	2.87 \pm 0.373	40.79 \pm 2.855*	1.75 \pm 0.158

* a regression line slope of negative direction

Lymphocyte proliferation was estimated by means of two methods: the immunocytochemical identification of Ki-67 expressing cells, and the cytogenetic estimation of replication indices (RI). The nuclear antigen Ki-67 appears on the nuclear membranes of proliferating cells only; it is absent on quiescent, non-proliferating cells [eg. 18, 19]. Thus, the estimation of Ki-67+ cells gives information about the cell fraction involved in the cell generation cycle (G₁, S, G₂ and M) and also about Ki-67-negative cells, i.e. the fraction of non-proliferating cells (G₀). The cytogenetic estimation of replication indices makes it possible to assess the rate of cell proliferation, since it differentiates between cells which replicated once (M₁), twice (M₂) and three times (M₃) in the presence of the thymidine analogue (BrdUrd; 30 μ M). The results lead to the calculation of the RI coefficient which is often used to calculate the average generation time of the culture [e.g. 20]. It was noted that among the tested compounds only AN slightly increased the RI of PHA-stimulated lymphocyte cultures. The other three compounds did not significantly change the rates of cell replication. On the other hand, all the tested compounds enlarged the fraction of Ki-67+ cells. We consider this to be a beneficial effect in terms of an immunomodulatory potency - it could extend the repertoire of lymphocytes able to recognize foreign antigens and proliferate in response to them.

The results indicate that the changes in the percentual representation of lymphocyte subpopulations might be caused by a recruitment of the cells to the proliferation cycle and by an enhanced expression of surface antigens on the cell membranes rather than by an increased proliferation rate of lymphocyte subpopulations. This conclusion can be supported, for instance, by examination of the B-cell subpopulation. In our experimental conditions (i.e. a culture time of 72h, the application of PHA-lectin as a mitogenic stimulator) it is impossible to obtain B-cell proliferation in the cultures. Thus, an increase in the B-cell percentual representation probably reflects an elevated number of the B-cells expressing their surface antigens. Summing up, our results suggest that the immunomodulatory action of the tested compounds could result in an increased number of lymphocytes which expressed the membrane antigens important to their immunosurveillance function.

This paper contains preliminary results indicating the immunomodulatory action of the tested compounds on human lymphocyte cultures, which was an additional activity to their antimutagenic action *in vitro*, described previously [1-7]. Since two of the compounds (anthocyanins and alkylresorcinols) are natural, relatively non-toxic products, they could be administered to people as regular diet components, both in order to forestall mutations and to modulate the immune system. Two synthetic compounds (todalazine and fluphenazine) should be chemically modified to obtain chemical derivatives which preserve the antimutagenic and immunomodulatory activities of the original drugs, but which have their main pharmacological action i.e. vasodilatation (todalazine) and antipsychotic, neuroleptic action (fluphenazine) reduced. However, even the original, unmodified drugs could be used in the treatment of a selected group of patients, for instance in the course of regular therapy of arterial hypertension (todalazine) or schizophrenic/paranoid psychoses (fluphenazine), especially in those patients who require antimutagens (e.g. heavy smokers) and/or additional stimulation of the immune system.

It is obvious that presented preliminary data on the immunomodulatory action of the tested antimutagens should be confirmed on a larger group of blood donors before the compounds are recommended to people as immunotropic drugs. We consider these results to be an indication that the antimutagenic action of the tested compounds could be utilised in parallel with their significant immunomodulatory activity.

REFERENCES

1. G¹siorowski, K., Szyba, K., Brokos, B., Ko³aczyńska, B., Jankowiak-W³odarczyk, M. and Oszmiański, J. Antimutagenic activity of anthocyanins isolated from *Aronia melanocarpa* fruits. **Cancer Lett.** 119 (1997) 37-46.

2. G¹siorowski, K., Brokos, B., Kozubek, A. and Oszmiański, J. The antimutagenic activity of two plant-derived compounds. A comparative cytogenetic study. **Cell. Mol. Biol. Lett.** 5 (2000) 171-190.
3. G¹siorowski, K., Szyba, K., Brokos, B. and Kozubek, A. Antimutagenic activity of alkylresorcinols from cereal grains. **Cancer Lett.** 106 (1996) 109- 115.
4. G¹siorowski, K., Szyba, K. and Urban, J. Todralazine influence upon the mutagenicity of some direct- and indirect-acting mutagens. **Mutat. Res.** 324 (1994) 133-137.
5. G¹siorowski, K. and Brokos, B. Evaluation of antimutagenic effect of todralazine in cultured lymphocytes. **Mutagenesis** 15 (2000) 137-141.
6. G¹siorowski, K. Antimutagenic activity of todralazine and fluphenazine. A comparison of the results from short-term cytogenetic tests. **Cell. Mol. Biol Lett.** 5 (2000) 397-403.
7. G¹siorowski, K., Brokos, B., Szyba, K. and Leszek, J. Antimutagenic activity of fluphenazine in the standard short-term tests. **Mutagenesis** 16 (2000) in press.
8. Gabrielska, J., Oszmiański, J., Komorowska, M. and Langner, M. Anthocyanin extracts with antioxidant and radical scavenging effect. **Zeitschr. Naturforsch.** 54c (1999) 319-324.
9. Oszmiański, J. and Sapis, J.C. Anthocyanins in fruits of *Aronia melanocarpa*. **J. Food Sci.** 53 (1988) 1241-1242.
10. Kozubek, A. and Tyman, J.H.P. Cereal grain resorcinolic lipids: mono and dienolic homologues are present in rye grains. **Chem. Phys. Lipids** 78 (1995) 185-198.
11. Kozubek, A. and Tyman, J.H.P. Resorcinolic lipids, the natural nonisoprenoid phenolic amphiphiles and their biological activity. **Chemical Rev.** 99 (1999) 1-26.
12. Hofman, F.M., Kanesberg, B. and Smith, D. Stability of T- and B-cell numbers in human peripheral blood. **Amer. J. Clin. Pathol.** 77 (1982) 710-711.
13. Perry, P. and Wolff, S. New Giemsa method for the differential staining of sister chromatids. **Nature** 251 (1974) 156-159.
14. Antoshina, M.N. and Poriadkova, N.A. A technique for differential staining of sister chromatids without using fluorochromes. **Cytol. Genet.** 4 (1978) 349-352.
15. Wranke, R. and Levy, R. Detection of T and B cell antigens with hybridoma antibodies. A biotin-avidin-horseradish peroxidase method. **J. Histochem. Cytochem.** 28 (1980) 771-776.
16. Hsu, S.M., Baine, L. and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. **J. Histochem. Cytochem.** 29 (1981) 577-580.

17. Campbell, M.J. Correlation and linear regression in: **Medical Statistic. A Commonsense Approach**, (Campbell, M.J. and Machin, D., Eds.), 2nd edition, John Willey and Sons, Chichester, New York, Brisbane, Toronto, Singapore, 1994, 87-104.
18. Key,G., Petersen,J.L., Becker,M.H.G., Duchrow,M., Schlueter,C. and Askaa,J. New antiserum against Ki67 antigen suitable for double immunostaining. **J. Clin. Pathol.** 46 (1993) 1080-1084.
19. Rose,D.S.C., Maddox,P.H. and Brown,D.C. Which proliferation markers for routine immunohistology. A comparison of five antibodies. **J. Clin. Pathol.** 47(1994)1010-1014.
20. Palma,V., Tudon,H., Buentello, L., Nava,S., Ostrosky,P. and Salamanca,F. Methods for analysis of cellular kinetics in PHA-stimulated blood lymphocytes using BrdUrd incorporation. **Mutat. Res.** 286 (1993) 267-273.