THE ANTIMUTAGENIC ACTIVITY OF TWO PLANT-DERIVED COMPOUNDS. A COMPARATIVE CYTOGENETIC STUDY.

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Abstract: The antimutagenic activity of alkylresorcinols from cereal grains and anthocyanins from Aronia melanocarpa fruit were compared in three short-term lymphocyte tests: a sister chromatid exchange test, a cytokinesis-blocked micronucleus assay and a thioguanine-resistance test. It was noticed that both tested compounds significantly decreased the rate and frequency of mutations induced in cultured lymphocytes with two standard mutagens: benz[a]pyrene and mitomycin C. Alkylresorcinols appeared to be more potent antimutagenic compounds than anthocyanins. Anthocyanins exhibited a stronger inhibitory effect on the generation and release of free radicals by human granulocytes in vitro, as measured with the NBT-reduction test. The results suggest that alkylresorcinols and anthocyanins exerted an antimutagenic influence through multifarious mechanisms, one of which could be a limitation of free radical involvement in mutagenesis.

Key Words: Alkylresorcinols, Anthocyanins, Antimutagenesis, Lymphocyte Short-Term Tests, Granulocyte Free Radicals

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INTRODUCTION

Antimutagenic agents are natural or synthetic compounds capable of lowering the frequency or rate of mutations by diverse modes of action [1-4]. Depending on their main mechanism, antimutagens can be classified into two general groups, i.e. desmutagens (countermutagens) which prevent DNA damage and fidelogens (bioantimutagens) which enhance repair fidelity of already existing DNA damage [3,4].

Modern analytical methods have proved that mutagenic agents are present in the human environment, often in relatively low doses, frequently below the detection level of standard chemical assays [5-7]. People are exposed to mutagens and carcinogens usually present in complex mixtures, such as urban air, cigarette smoke, and water contaminants [8]. The components of complex mixtures may interact with one another, which modulates mutagenic activity [7]. The final effect of interactions in such a mixture is difficult to predict. That is why the opinion prevails that humans inevitably, though often unwittingly, are and will be exposed to mutagenic agents, and that a total elimination of mutagenic agents from the human environment is impossible [e.g. 4, 5]. Since people are doomed to live surrounded by mutagens, the genotoxic effects of mutagenic and carcinogenic agents must be reduced by regular intake of antimutagenic compounds. The best candidates appear to be natural diet components, which could ensure an ample delivery of natural antimutagens sources during daily meals. The majority of antimutagen from natural sources are derived from the plant kingdom. A vast body of evidence proves that many foods contain components that exert antimutagenic and cancer chemopreventive actions [e.g. 9-14].

A number of large, multicenter projects are implemented to extract, isolate and chemically identify antimutagenic compounds from plant material [e.g. 3, 10, 14]. This branch of antimutagenesis and cancer chemoprevention has developed rapidly and broadly over the last decade, resulting in the discovery of new antimutagenic compounds as well as specifying mechanisms of their action [2, 10, 11, 13].

We described previously the antimutagenic activity of two plant-derived compounds: alkylresorcinols (AR) from cereal grains [15] and anthocyanins (AN) from Aronia melanocarpa fruit [16]. AR and AN were separated and purified chromatographically, and subsequently characterized with the standard chemical spectroscopic methods [17-21].

The general chemical formula of the tested compounds is given in Fig. 1.

C 19 saturated aliphatic chain alkylresorcinol, shown in Fig. 1A, is a typical member of resorcinolic lipids from the bran milling fraction. Cyanidin 3-O galactoside, presented in Fig. 1B, predominates in the glycosidic flavonoid fraction extracted from Aronia melanocarpa fruit.
In the Ames test, we noticed that the effect of the standard mutagenic agents was considerably decreased in the presence of both AR [15] and AN [16]. The antimutagenic action of the tested compounds was also confirmed by the initial screening studies with the sister chromatid exchange (SCE) test in cultured human lymphocytes [15, 16]. However, further experiments were necessary to estimate and explain the mechanisms of AR and AN action on mutagenesis in human lymphocyte cultures. The aim of the present study is to verify and compare the antimutagenic activities of AR and AN against some standard mutagens in human lymphocyte cultures by means of three different end-point mutagenicity tests: the thioguanine-resistance test (TG-R), the cytokinesis-blocked micronucleus assay (CBMN), and the sister chromatid exchange test (SCE). The influence of AR and AN on the generation of free radicals in vitro by human blood-derived granulocytes is also presented.

MATERIALS AND METHODS

Chemicals
Benzo[a]pyrene (B[a]P), mitomycin C (MMC), cytochalasin B, 5-bromo-2'-deoxyuridine (BrdUrd), phorbol 12-myristate 13-acetate (PMA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, USA). Blood cell-separation solutions: Histopaque-1077, Histopaque-1119, as well as components of cell culture media: RPMI 1640, foetal calf serum (FCS) and L-glutamine were also obtained from Sigma (St. Louis, USA).
Phytohemagglutinin (PHA-M) was obtained from Gibco (Gaithersburg, USA). The stains: acridine orange, ethidium bromide, azur II, eosine B, trypan blue, Giemsa-solution and nitroblue tetrazolium (NBT) were purchased from Sigma (St. Louis, USA). The other reagents used for buffers and culture media preparation were from POCH (Gliwice, Poland).

Alkylresorcinols
AR (5-n-alk(en)y1resorcinols) were isolated and purified chromatographically from the acetone extract of a rye bran milling fraction according to the procedure previously described [18]. The structure of the isolated material was established by means of UV, IR, proton-NMR and MS analyses and compared to synthetically obtained 5-n-pentadecyl-, 5-n-nonadecyl- and 5-n-heneicosylresorcinols [17]. Quantitative determinations of AR were done by the colorimetric method [18]. It was established that this isolation procedure provided 5-n-alk(en)y1resorcinols with a mostly saturated aliphatic chain from C15 to C27; monoenoic and dienoic AR comprised 15 - 20% of the total extract and the configuration of all double bonds was determined to be at the 8- and 11-position of the aliphatic chain [17]. The molecular weight of AR from such a preparation was established as being close to 376.33. The lyophilized AR was stored in a freezer. On the day of the experiment it was dissolved with DMSO, and filtered through a 0.2 μm Milipore PTFE filter (Sartorius, Germany). Serial dilutions were prepared and AR-solution was added to the cell culture at the volume of 50 μl at the dose needed to obtain the defined concentration in the culture medium (concentration range: 0.78 - 12.5 μg/ml).

Anthocyanins
Anthocyanins were isolated from fruits of Aronia melanocarpa by extraction with water containing 200 ppm of SO₂. The extract was adsorbed on Purolite AP 400 resin (Purolite, United Kingdom) for further purification [20]. The anthocyanins were then eluted out with 80% ethanol, concentrated and lyophilized. The detailed description of this procedure is included in Polish Patent N° PL158707. By means of the above procedure a mixture of four anthocyanins was obtained. The proportion of the components was established by analysing the HPLC separation records at 520 nm [20].
It was calculated that cyanidin 3-0 galactoside was 65.5%, cyanidin 3-0 glucoside 29.8%, cyanidin 3-0 xyloside 3.2% and cyanidin 3-0 arabinoside 1.5% of the anthocyanin mixture. Since the content of monohexose anthocyanidins (i.e. cyanidin 3-0 galactoside and cyanidin 3-0 glucoside) constituted 95.3% of the total mixture and the molecular weight of these two components was identical, estimated as 449.40, the molecular weight of the whole AN preparation can be assumed as being closely approximate to 449.40. The lyophilized AN powder was stored in a freezer. The freshly prepared water
solution of AN was filtered through a 0.2 µm Milipore hydrophilic filter (Sartorius, Germany), and added to lymphocyte cultures to reach the assumed final concentration (1.562 - 25.0 µg/ml).

**Blood cell separation**
Heparinized blood was obtained by venipuncture from three healthy male volunteers aged 45-50, smoking 20-30 cigarettes/day. The cells were separated by a single-step discontinuous density-gradient centrifugation technique with Histopaque-1119 and Histopaque-1077 layers [22]. Isolated lymphocytes and granulocytes were washed in the phosphate buffer saline (PBS, pH 7.2) and intended for biological tests.

**Sister chromatid exchange test (SCE)**
Lymphocytes were cultured in 24-well plastic dishes at a density of 5 x 10^5 cells/ml in complete culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine) and stimulated to mitogenesis with a PHA-M (1% v/v) for 72 h. The tested mutagens: B[a]P (dissolved in DMSO) and MMC (dissolved in bidistilled water) were added to the culture at the volume of 50 µl to obtain the desired final concentration (B[a]P - 16 µM; MMC - 0.02 µg/ml). The tested solutions of AN and AR were added to the culture simultaneously with the tested mutagens. The thymidine analogue, bromodeoxyuridine (BrdUrd), was added for the last 48 hours of the culture time to the final concentration of 30 µM. The cultures were harvested following the standard cytogenetic method. The cell smears on glass slides were air-dried for 3 days. Afterwards, the slides were immersed in 2 x SSC solution (0.03 M sodium citrate in a 0.3 M sodium chloride-aqueous solution) at 62°C for 30 min and, simultaneously, they were illuminated under a UV-lamp [23]. Then the slides were stained with a mixture of dyes (azur II / eosin B) to reveal the differential chromatid staining [24] and examined under a microscope. The results comprised a mean SCE/metaphase among 25 harlequin metaphases, calculated in each of the analysed slides.

**Cytogenetic assessment of lymphocyte proliferation**
The replication indices of lymphocyte cultures were estimated by counting under a microscope the number of metaphases in the first (M₁), second (M₂) and third (M₃) division in the presence of BrdUrd and calculated according to the formula RI = (M₁+2M₂+3M₃) / 100.
The mitotic indices (MI) were determined in each culture's slide within 1000 cells found randomly during the microscope examination. The proliferation potential (PP) was calculated by multiplication of the mitotic index and the replication index in each lymphocyte culture; PP = MI x RI.
Cytokinesis-blocked micronucleus assay (CBMN)
The lymphocyte micronucleus assay was performed by means of cytokinesis-blocked assay in accordance with the standard procedures [25, 26]. Briefly, lymphocyte cultures were stimulated with lectine PHA-M (1% v/v), and the standard elastogenic agent, mitomycin C (MMC), which induces micronuclei, was added to the culture medium to make the final concentration of 0.02 μg/ml. AN and AR were added to the cultures at a volume of 50 μl in a dose making up the concentration needed. The control cultures contained the same volume of water or DMSO respectively. The MMC and AN or AR were present in culture medium for the whole culture period, i.e. for 72h. After 24h of culture, lymphocytes were exposed to a cytokinesis-blocking agent, cytochalasin B, added to the final concentration of 4.5 μg/ml, for the last 48h. The cultures were harvested according to the method given in the literature [26] with mild hypotonic treatment (0.075M KCl for 10 min.) followed by fixation with cold methanol : acetic acid (3:1). The cell suspensions were gently spread on microscopic slides, dried and stained with 10% Giemsa solution (in phosphate buffered saline, pH 6.8) for 5 min. The slides were examined under a microscope and the number of micronuclei was counted within 1000 binucleated lymphocytes.

Thioguanine - resistance assay (TG-R)
Lymphocytes were suspended in culture medium and stored in a plastic culture bottle for 18h at 4°C to prevent phenecopies. Afterwards, the cells were suspended in a fresh medium and cultured for 48h according to the assay method given in the literature [27, 28], with small modifications. Briefly, 0.8 x 10⁶ cells/ml were cultured in 24-well plastic dishes and stimulated with PHA-M (1% v/v); with the presence of 2 x 10⁴ M of thioguanine (TG). The mutagen B[a]P (16 μM) and the tested AN or AR solutions were added at the cultures’starting point. After 24h bromodeoxyuridine (BrdUrd; 30μM) was added to the cultures for the next 24h. The harvesting procedure comprised centrifugation of the cultures, fixation of pellets with 75% ethanol for 30 min., and treatment with a lysis solution (containing: RNA-se, 0.2 mg/ml; 0.5 mM disodium versenite and 0.5% non-ionic detergent Nonidet NP- 40) for 30 min. at 10°C. For partial denaturation of DNA the cell pellets were suspended with 0.5 N HCl and stored for 30 min. at room temperature. Then the cells were washed twice with phosphate buffered saline (PBS) and smears were prepared on microscopic slides. Cells which had incorporated BrdUrd into the DNA were detected by means of the immunocytochemical procedure with monoclonal mouse antibody, able to recognise BrdUrd in single stranded DNA (clone Bu20a, DAKO, Denmark). Visualisation and staining were performed following the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [29-31]. Following the above procedure thioguanine-resistant lymphocytes stain
intensively red. They were counted within 5000 cells randomly found under the microscope.

Estimation of granulocyte superoxide radicals
The influence of AN or AR on superoxide radical generation by human granulocytes in vitro, was evaluated with the routine nitroblue tetrazolium (NBT)-reduction test [32]. In the test a soluble NBT penetrates the cells and becomes converted by intracellular (preferentially superoxide) free radicals to blue-black formazan, which precipitates intracellularly. The NBT-reduction test was carried out both in the presence and in the absence of the standard granulocyte stimulator (PMA; 100 ng/ml) and, separately, in the presence of the promutagen (B[a]P; 16 μM). Granulocytes were suspended to the density of 2 x 10^6 cells/ml in phosphate buffered saline, pH 7.2, containing NBT (final concentration of 0.2%), and the desired concentration of AN or AR and and PMA or B[a]P. After the incubation of samples for 45 min. at 37°C in a shaking water bath, the reaction was terminated and the cells were centrifuged at 4°C. The pellets were then lysed with dimethylformamide at 56°C in a shaking bath and absorption (A_{515nm}) was measured with a spectrophotometer in relation to cell-free blank samples.

Statistical analysis
For statistical examination of the results, the regression analysis of dose-response relations and the multifactor analysis of variance (MANOVA) were chosen [e.g. 33].
In regression analysis the concentrations were recalculated and given as ln(x+1), where x = concentration of AR or AN. The experimental results were expressed in proportion to relative controls [E/E_0]. In the semilogarythmic scale, the dose-response relations could be described by linear regression lines. The main purpose of the analysis was to estimate the regression line slope (a_1) and to assess if the slope was significantly different from zero. It could prove that the observed effects of AR and AN were significantly dose-related.
The multifactor analysis of variance (MANOVA) was performed in order to estimate the significance of dose-response relations as well as for evaluation of the differences between various test-systems in detection of the AR and AN antimitogenic action.
Both regression equations and MANOVA were computed by means of the Systat software (Systat, Evanston, USA).

RESULTS AND DISCUSSION
The effect of AR and AN on the viability of human blood-derived lymphocytes in 4- and 18-hour cultures was estimated by means of the standard trypan-blue exclusion test and the double fluorescence staining with an acridine orange /
ethidium bromide mixture. It was observed that AR was not cytotoxic to human lymphocytes up to the concentration of 12.5 μg/ml, whereas at higher concentrations the number of dead cells markedly increased. In the case of AN, the percentage of dead cells increased at concentrations of 50 μg/ml and higher. The number of dead cells did not rise significantly in the presence of the tested mutagens, B[a]P (16μM) or MMC (0.02 μg/ml), added to the culture medium simultaneously with AR or AN. Therefore, the upper level of the tested compound concentrations applied in the cytogenetic test was 12.5 μg/ml and 25 μg/ml for AR and AN, respectively.

The influence of AR and AN on the decrease of lymphocyte proliferation caused by tested mutagens was evaluated in cultures initially stimulated to mitogenesis with PHA-M (1% v/v). It was observed that AR decreased the proliferation potential (PP) of lymphocytes cultured in the presence of B[a]P (16μM) and MMC (0.02 μg/ml). The effect depended on the AR concentration and was especially strong at the highest tested concentration (12.5 μg/ml). At this concentration, the PP was lowered by more than 75% in the cultures in which B[a]P was applied and by about 92% in the cultures where MMC was added. To quantify the dose-effect relation of AR in the lymphocyte cultures, we calculated the linear regression equations. The slopes of regression lines were of negative direction and significantly different from zero both in the case of B[a]P + AR \[y = 1.01 - 0.28 \ln(x+1); r = 0.96; F = 47.96; p = 0.0023\] and in the case of MMC + AR \[y = 0.998 - 0.316 \ln(x+1); r = 0.97; F = 63.91; p = 0.0013\]. It proved that AR significantly lowered the PP of lymphocytes cultured in the presence of the mutagens.

The impact of AN on the PP of lymphocytes cultured in the presence of mutagens was different from that of AR. At the lower concentrations of AN, the proliferation was enhanced by 13 - 20% with MMC and by 7 - 10% with B[a]P. At the highest concentration of AN (25 μg/ml), a small decrease of PP was observed both in MMC-containing cultures (by 15%) and in B[a]P-treated cultures (by 20%). Calculated regression equations showed that the effect of AN on the PP was neither significant in cultures with MMC (F = 2.57; p = 0.186; NS) nor in cultures where B[a]P was present (F = 5.25; p = 0.084; NS). The results proved that AN did not significantly lower the proliferation rate of the mutagen-treated cultures, and at lower AN concentrations even a noticeable increase of cell proliferation appeared.

Although the relationships between proliferation and genotoxicity seem to be complex [e.g. 34, 35], a commonly accepted opinion is that a decrease in the cell proliferation rate would facilitate detoxication and repair of damage caused by mutagenic agents [36-38]. The cells exhibiting lower proliferation kinetics are believed to have relatively more time to induce the defense systems, such as glutathione S-transferases, cytochrome P450 monoxygenases and DNA repair enzymes [35, 36]. In the light of the arguments presented above, the general
conclusion from our studies is that the observed diminution of mitogenesis by AR in mutagen-damaged lymphocyte cultures should be perceived as an effect beneficial for detoxication and repair processes. AN, on the contrary, did not exert this influence.

MMC belongs to the group of direct-acting mutagens, whereas B[a]P to indirect-acting (promutagens). This means that initial activation by intracellular enzymatic systems is the fundamental step in the B[a]P genotoxicity. Such an activation is not necessary for MMC to manifest its genotoxic effect. In the genotoxicity tests with indirect-acting mutagens, a rat liver microsomal fraction is usually added in order to activate the promutagen to genotoxic derivatives [39]. For instance, the addition of a microsomal fraction is necessary in genotoxicity testing with mammalian cell lines \textit{in vitro}. However, it is not essential in tests with freshly blood-derived lymphocytes, since their own microsomal system is still sufficient to activate B[a]P to genotoxic derivatives \textit{in vitro} [40, 41]. Therefore, in our experiments with B[a]P we did not apply an external microsomal fraction.

The lymphocytes used in evaluation of the impact of AR and AN were initially characterised by short-term \textit{in vitro} tests, the results of which are presented in Tab.1.

\begin{table}
\centering
\begin{tabular}{lcccc}
\hline
& \multicolumn{2}{c}{Negative control} & \multicolumn{2}{c}{Positive control} \\
Test: & x ± SD; n = 3 & B[a]P (16 μM) & x ± SD; n = 3 & MMC (0.02 μg/ml) \\
\hline
PP & 12.2 ± 3.41 & 6.3 ± 2.31 & 3.2 ± 1.09 & \\
SCE & 13.4 ± 4.22 & 38.3 ± 3.69 & 50.4 ± 6.14 & \\
TG-R & 12.6 ± 6.23 & 82.7 ± 16.52 & & \\
CBMN & 1.2 ± 0.36 & & 4.6 ± 1.86 & \\
\hline
\end{tabular}
\caption{Introductory characteristic of lymphocyte cultures in four short-term \textit{in vitro} tests. Negative control cultures contained neither mutagen nor AR and AN. Positive control cultures contained the mutagen: benzo[a]pyrene - B[a]P or mitomycin C - MMC.}
\end{table}

PP – proliferation potential (MI x RI); SCE – sister chromatid exchange test (mean frequency of chromatid exchanges per mitosis); TG-R – thioguanine resistance test (frequency of resistant cells x 10^6); CBMN – cytokinesis blocked micronucleus assay (% of binucleated lymphocytes containing micronuclei).
The blood donor volunteers were three healthy male smokers (20 or more cigarettes per day) aged 45 - 50. As may be seen in Tab. 1, lymphocytes from the negative control cultures (without mutagen and AR or AN) exhibited a relatively high level of background genotoxic damage in the TG-R, CBMN and SCE tests.

The addition of B[a]P and MMC caused a marked increase in genotoxic damage. B[a]P caused a 3-fold increase in the mean SCE frequency in cultured cells, a 6-fold increase in TG-R cell numbers and a decrease in the PP of more than 50%. MMC caused a 4-fold increase in SCE frequencies, an almost 4-fold increase in CBMNIs and a decrease in the lymphocyte proliferation rate (PP) of more than 70%. The high level of background mutations could be explained, at least in the most part, by the smoking habit of the tested blood donors.

We estimated that AR and AN did not significantly change the level of background mutations in the three applied cytogenetic tests (data not provided). The estimations proved that AR and AN did not reveal a genotoxic activity in our experimental conditions.

The impact of AR and AN on the mutagenicity of the standard mutagens - B[a]P or MMC - on human lymphocytes in vitro is shown in Fig. 2 in the form of linear regression lines. The relative regression equations are given in Tab. 2.

Fig. 2. The impact of AR (left) and AN (right) on the mutagenicity of standard mutagens: B[a]P (16 μM) and MMC (0.02μg/ml) on human lymphocytes. The results obtained in the presence of the standard mutagen and AR or AN (E) are compared to the relative control cultures (E₀), i.e. lymphocytes cultured in the presence of the mutagen only. The dose-dependent effects were calculated with regression equations.
Tab. 2. Regression analysis of the data given in Fig. 2.

<table>
<thead>
<tr>
<th>Test:</th>
<th>$a_0$</th>
<th>SE</th>
<th>$a_1$</th>
<th>SE</th>
<th>$r$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCE(MMC)</td>
<td>1.058</td>
<td>0.0516</td>
<td>-0.098</td>
<td>0.0339</td>
<td>0.82</td>
<td>0.0442</td>
</tr>
<tr>
<td>SCE[B[a]P]</td>
<td>0.992</td>
<td>0.0243</td>
<td>-0.084</td>
<td>0.0159</td>
<td>0.93</td>
<td>0.0063</td>
</tr>
<tr>
<td>CBMN(MMC)</td>
<td>1.029</td>
<td>0.0416</td>
<td>-0.130</td>
<td>0.0252</td>
<td>0.95</td>
<td>0.0141</td>
</tr>
<tr>
<td>TG-R[B[a]P]</td>
<td>0.976</td>
<td>0.0386</td>
<td>-0.202</td>
<td>0.0233</td>
<td>0.98</td>
<td>0.0032</td>
</tr>
<tr>
<td>SCE(MMC)</td>
<td>0.993</td>
<td>0.0141</td>
<td>-0.039</td>
<td>0.0070</td>
<td>0.94</td>
<td>0.0050</td>
</tr>
<tr>
<td>SCE[B[a]P]</td>
<td>0.935</td>
<td>0.0412</td>
<td>-0.105</td>
<td>0.0205</td>
<td>0.93</td>
<td>0.0069</td>
</tr>
<tr>
<td>CBMN(MMC)</td>
<td>0.974</td>
<td>0.0410</td>
<td>-0.158</td>
<td>0.0204</td>
<td>0.97</td>
<td>0.0015</td>
</tr>
<tr>
<td>TG-R[B[a]P]</td>
<td>0.890</td>
<td>0.0791</td>
<td>-0.157</td>
<td>0.0393</td>
<td>0.92</td>
<td>0.0281</td>
</tr>
</tbody>
</table>

All the mutagenicity data are expressed as an $E/E_0$ ratio: the proportion of the results obtained in the cultures containing both AR and the standard mutagen (E) to the results obtained in the cultures containing only the mutagen (positive control, $E_0$, the same as given in Tab. 1). The regression equations show the dose-response relations of the observed effect.

As may be seen in Fig. 2 (left), AR lowered the mutagenicity of the standard mutagens - all the regression lines are of negative direction and their slopes are significantly different from zero.

As the regression equations prove, the antimitogenic action of AR was relatively strongest in the TG-R test; it was about 1.5-times as strong as in the CBMN assay, and more than twice as strong as in both SCE tests.

It should also be noticed that in the SCE tests the action of AR was of similar strength in the case of both mutagens. This suggests that the mechanism of AR antimitogenic actions probably involves more processes than simply the inhibition of microsomal B[a]P-activating enzymes, since the mutagenic action of both MMC (direct mutagen) and B[a]P (promutagen) was diminished by AR to the same extent.

Taking into consideration the different end-points of the applied lymphocyte short-term tests, one can conclude that AR effect was the strongest in the test which detects point mutations (TG-R test), moderately strong in the test which detects clastogenic and aneuploidogenic action of mutagens (CBMN assay), and the weakest in the test which reveals rearrangements and rejoining of DNA strands (SCE test).

As may be seen in Fig. 2 (right), AN decreased the mutagenicity caused by the applied mutagens. The regression lines are of negative direction and their slopes are significantly different from zero. The antimitogenic action of AN was
almost equally strong in the CBMN assay and TG-R test, whereas in comparison to them the effect of AN was weaker by about 35% in the SCE test with B[a]P and by 75% in the SCE test with MMC. In the SCE test with the MMC, the impact of AN was very weak - the slope of the regression line is mild, although statistically significant. In comparison to that result, the slope of regression line in the SCEs test with B[a]P is almost three times as steep. It suggests that AN action consists largely of inhibiting microsomal, enzymatic activation of B[a]P. We concluded that the mechanisms of AN antimutagenicity primarily involved decreasing the clastogenic/aneuploidogenic action of the mutagens (CBMN assay) and lowering point mutation frequencies (TG-R test).

The analysis of variance confirmed that the antimutagenic effects of AR and AN significantly depended on their concentrations in lymphocyte cultures (in the case of AR: F = 234.25, df = 3, p < 10^-5; and in the case of AN: F = 176.61, df = 3, p < 10^-5). It also proved that the applied tests differed significantly in their description of AR and AN antimutagenic action (F = 304.86, df = 3, p < 10^-5; and F = 743.70, df = 3, p < 10^-8, respectively).

Since the applied tests were able to detect the different end-points of mutagenicity, the differences between the tests suggested that various mechanisms of AR and AN antimutagenic action unequally contributed to the final antimutagenic effect. Therefore, further attempts at comparing specific mechanisms of the antimutagenic action of AR and AN were justified.

For this purpose, we compared the data presented in Fig. 2, calculating the antimutagenic specific activity (ASA) coefficient. The decrease by AR or AN of the mutagens' genotoxicity quantified with regression equations (Fig. 2) was referred to the molar concentration of AR or AN in a culture medium. The assumed molecular weight was 376.33 in case of AR and 449.40 in case of AN. The ASA coefficients can be perceived as a percentual decrease of mutagenicity calculated per 1 mM of AR and AN added to the culture medium. The results of such calculation are given in Tab. 3.

Tab. 3. The antimutagenic specific activity (ASA) of AR and AN.

<table>
<thead>
<tr>
<th>Test</th>
<th>AR</th>
<th>AN</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCE (MMC)</td>
<td>9.09 ± 0.393</td>
<td>1.73 ± 0.132</td>
</tr>
<tr>
<td>SCE (B[a]P)</td>
<td>7.81 ± 0.252</td>
<td>4.66 ± 0.318</td>
</tr>
<tr>
<td>CBMN (MMC)</td>
<td>12.09 ± 0.784</td>
<td>7.02 ± 0.512</td>
</tr>
<tr>
<td>TG-R (B[a]P)</td>
<td>18.80 ± 0.946</td>
<td>6.98 ± 0.659</td>
</tr>
</tbody>
</table>
As may be seen in Tab. 3, the ASA coefficients are markedly higher for AR than for AN: about 1.7 times higher in the SCE test with B[a]P and in the CBMN assay, almost 3 times higher in the TG-R test and more than 5 times higher in the SCE test with MMC.

The ASA coefficients of AR and AN differ considerably in various tests of mutagenicity carried out with the same mutagen. In the SCE test with AN the MMC mutagenicity decreased by 1.73% per 1mM of AN, whereas in the CBMN assay with the same mutagen a 7.02% decrease was noticed. Also in case of AR, the ASA coefficients differed significantly in both tests with MMC, being almost 1.35-times as high in the CBMN assay as in the SCE test. The differences noticed in the compared tests may suggest different mechanisms of AN and AR antimutagenic action, probably more complex than simply interception and inactivation of MMC.

The highest ASA coefficients in Tab. 3 are those of AR in the TG-R test and in the CBMN assay. The corresponding ASA coefficients of AN are 2.69- and 1.72-times lower. The general conclusion drawn from the data in Tab. 3 is that AR appears to be a significantly more potent antimutagen than AN.

The activation of promutagens to their genotoxic derivatives is a complex pathway mediated by microsomal mixed-function oxidases and other enzyme systems that may involve formation of free radicals [41-43]. Furthermore, several mutagenic compounds can enhance generation of cellular free radicals, which may cause an excess of radicals in the cell and intensify DNA damaging processes [42, 44]. Therefore, a putative antimutagenic compound should be tested in the aspect of its influence upon cellular free radical systems.

We assessed the impact of AR and AN on human granulocyte free radicals level by means of the NBT-reduction test in vitro, both in the presence and in the absence of the standard stimulator of free radical generation (PMA) or the mutagen (B[a]P).

Initial spectrophotometric estimations of the free radical level (NBT-reduction test) in control granulocyte samples supplied the following results (x ± SD, n = 3): (1) in samples containing the B[a]P (16 μM): A515nm = 0.084 ± 0.0221; (2) in samples containing the PMA (100 ng/ml): A515nm = 0.122 ± 0.0131; (3) in samples containing the solvent only (DMSO, 10 μl): A515nm = 0.066 ± 0.0205. The data given above were considered as the control base-level (E0) to which all the results of AR or AN impact were compared (E/E0). The results describing the impact of AR and AN on free radical generation are presented in Fig. 3 and corresponding Tab. 4.

An initial impression from Fig. 3 is that AN revealed a more potent inhibitory action on granulocyte free radicals than AR did - the plots of regression lines describing the effect of AN (right) are generally steeper than those of AR (left). Both of the tested compounds lowered the level of “spontaneously” generated radicals (without PMA and B[a]P), although the slope of the regression line describing the effect of AN was almost 2.5 times as high as that of AR.
Fig. 3. Influence of AR (left) and AN (right) on the free radical level in human granulocytes in vitro assessed with the NBT-reduction test. The AR or AN effect on the spontaneous generation of free radicals was estimated in granulocyte samples in which only AR or AN were present (E) and were compared to the free radical level in granulocyte samples containing solvent only (E₀). The impact of AR or AN on the radical level was estimated in the presence of B[a]P (16μM) and PMA (100ng/ml), and was compared to the E₀ level established in granulocyte samples containing B[a]P or PMA only. The dose-response effects were calculated with regression equations.

Tab. 4. Regression analysis of the data given in Fig. 3.

<table>
<thead>
<tr>
<th>Compounds:</th>
<th>a₀</th>
<th>SE</th>
<th>a₁</th>
<th>SE</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>1.007</td>
<td>0.0101</td>
<td>-0.042</td>
<td>0.0050</td>
<td>0.979</td>
<td>0.0036</td>
</tr>
<tr>
<td>PMA + AR</td>
<td>0.982</td>
<td>0.0259</td>
<td>-0.084</td>
<td>0.0129</td>
<td>0.956</td>
<td>0.0029</td>
</tr>
<tr>
<td>B[a]P + AR</td>
<td>1.009</td>
<td>0.0241</td>
<td>-0.021</td>
<td>0.0120</td>
<td>0.660</td>
<td>NS</td>
</tr>
<tr>
<td>AN</td>
<td>1.090</td>
<td>0.0779</td>
<td>-0.104</td>
<td>0.0308</td>
<td>0.889</td>
<td>0.0434</td>
</tr>
<tr>
<td>PMA + AN</td>
<td>1.029</td>
<td>0.0293</td>
<td>-0.135</td>
<td>0.0116</td>
<td>0.986</td>
<td>0.0003</td>
</tr>
<tr>
<td>B[a]P + AN</td>
<td>1.008</td>
<td>0.0237</td>
<td>-0.039</td>
<td>0.0093</td>
<td>0.901</td>
<td>0.0143</td>
</tr>
</tbody>
</table>

In the samples containing granulocytes stimulated with PMA, the inhibitory effects of the tested compounds were considerable, although the slope of the regression line was 1.6 times as high for AN as for AR.
The impact of AN on the level of free radicals in the presence of B[a]P was mild but statistically significant, whereas in the case of AR the effect was not significant. The analysis of variance proved that the effects on free radical generation depended strongly on the test-system ("spontaneous", PMA-stimulated and B[a]P-stimulated free radical generation) and the differences between the test systems in description of the effect of the applied antimutagens were especially strong in the case of AN. The following F-statistics were computed: F = 88.52, df = 2, p < 10^{-5} and F = 1283.73, df = 2, p < 10^{-5} for AR and AN effects, respectively.

The analysis also confirmed that the effect of AR and AN on the generation of free radicals by human granulocytes in vitro significantly depended on the concentration of the tested compounds (in the case of AR: F= 33.39, df = 3, p < 10^{-5} and in the case of AN: F = 1265.95, df = 3, p < 10^{-5}). It should be noticed that the F-statistics were markedly higher for AN than for AR. It may confirm that the influence on the free radicals generation was significantly more potent in the case of AN than in the case of AR.

It has been established that in rat liver microsomes in vitro, a significant part of B[a]P is transformed into the form of free radicals, which causes DNA adducts [43, 45] and adds their genotoxic action to the majority of adducts caused by diol epoxides, the main genotoxic derivatives of this mutagen [46, 47]. Our results suggest that AN is able to decrease the level of radicals in the presence of B[a]P, while AR cannot exert such an action.

The activation of a granulocyte oxidative burst by PMA and other phorbol esters is a consequence of phorbol esters' binding and activating the protein kinase C [48-50]. Since the protein kinase C is a mediator of the major cellular signal transduction pathway, its high affinity to phorbol ester binding could explain a broad range of phorbol esters' biological activities, such as the influence on the cellular growth control, carcinogenesis and tumor promotion [48, 51]. The inhibitory action of AN and AR on the granulocyte free radical system stimulated with PMA may suggest an interference of the tested compounds with this signal transduction pathway, also with those involved in tumor promotion and carcinogenesis. This hypothesis has still to be evaluated.

Summing up, we can conclude that AR exhibited significantly stronger antimutagenic activity than AN did, whereas AN was a markedly more potent inhibitor of free radical pathways. We consider our results to be an indication that the mechanisms of AR antimutagenic action are multifarious and more complex than simple inhibition of free radical processes. The main mechanism of the AN antimutagenic effect seems likely to be connected with the inhibition of free radical-dependent mutational events. The search to elaborate on AR and AN antimutagenic action mechanisms is currently in progress.

Considering a possible relevance of our results to humans, it should be remembered that the prediction of effects on human health on the basis of in
vitro assays is a complex issue, which has been debated for decades and still remains controversial [e.g. 52, 53]. Since alkylresorcinols and anthocyanins are common food components, the first issue is their enteric absorption after oral intake. In the literature available, we have found only one paper describing absorption of alkylresorcinols from rats' bowels after a single oral dose of [4-\(^{14}\)C]-5-n-heneicosylresorcinols isolated from corn oil [54]. The estimated radioactivity of urine, faeces and blood covered about 80% of the total \(^{14}\)C - input. It showed that alkylresorcinols are absorbed from the bowels after oral administration.

In case of anthocyanins, the HPLC analysis detected cyanidin 3-0-glucoside and cyanidin 3-0-sambubioside in human plasma at two hours after drinking elderberry juice [55]. In case of people suffering from various types of cancers, diet supplementation with juice from *Aronia melanocarpa* resulted in significant immunocorrectory effects, which can be most probably related to anthocyanins present in the juice [56, 57].

The papers cited above directly prove that circulating blood cells are exposed to enteric-absorbed alkylresorcinols and anthocyanins. However, the detailed study of AR and AN uptake, pharmacokinetics and pharmacological activity in humans should be a subject of separate future research.

REFERENCES


