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In Vitro Antileukemic, Antioxidant and Prooxidant Activities of Antoksyd S (C/E/XXI): a Comparison with Baicalin and Baicalein

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Abstract. *There is increasing interest concerning the use of natural antioxidants as low toxic antileukemic compounds. Antoksyd S (C/E/XXI), is a novel herbal drug derived in Poland from the powdered roots of Scutellaria baicalensis, and the biological activities of its major components (baicalin and baicalein) were compared on the human leukemia cell line HL-60. On MTT assay, Antoksyd S (C/E/XXI) showed an obvious cytotoxic effect on HL-60 cells, which was compared with those caused by cisplatin and doxorubicin under the same experimental conditions. A comparative assay of the antioxidative and prooxidative capacity of Antoksyd S (C/E/XXI) was also undertaken using two different reactive species: superoxide ($O_2^{\bullet -}$) and a transition metal (Cu^{2+}). Antoksyd S (C/E/XXI) has low toxicity, acting as a modifier of HL-60 cells proliferation and as an antioxidant, which could act prooxidatively in the presence of transition metal ions. Taken together, it seems reasonable to suggest that Antoksyd S (C/E/XXI) as compared to baicalin and baicalein, or to the cytostatics cisplatin and doxorubicin, might be an especially good candidate for the future development of new therapeutic techniques, alone or in "combination treatment regimens", to enhance leukemia cell killing.*

Reactive oxygen species (ROS) play an important role in carcinogenesis, in all cancer stages (initiation, promotion and progression) and in oncogenes or carcinogens activation (1-6). ROS cause DNA fragmentation, activation of cell defense mechanisms, NAD depletion and apoptosis (1, 2, 6-9). Cancer development and progression are often associated with defective, reduced activities of the cell defense (antioxidant) systems. Hence, the concept of systematically administered, low toxicity antioxidants is appealing (6, 10, 11-13). Suitable antioxidants should be able to easily cross the blood-brain barrier and, when present at low concentrations, should significantly delay or prevent the oxidation of substrates such as carbohydrate, lipid, DNA or protein (6). Among natural products, flavonic antioxidants from plants may prevent and/or combat the ROS-induced pathological status in cancer (2, 4, 6, 10, 13).

In recent years there has been increasing evidence that the flavone polyphenolics from *Scutellaria baicalensis*, baicalein (BAIN, 5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) and baicalin (BA, baicalein-7-β-D-glucopyranosiduronic acid) (Figure 1), act as inhibitors of tumor cell proliferation and as potent inducers of apoptotic death (14-18). It has been reported that BA and BAIN protect cells from oxidative stress (19-21) through their antioxidant activity (12, 14, 22-26). Controversially, it has been suggested that they might display cytotoxicity through prooxidant action (17, 27, 28) including the formation of radicals.

The design of the novel herbal drug, Antoksyd S (C/E/XXI) (Ant S) from *Scutellaria baicalensis* (roots) (Patent No 319735, Polish Patent Office, January 2004, Warsaw, Poland) aimed to achieve a combination of its major components BA and BAIN (Figure 1), so as to overcome the drawbacks of both flavonoids relating to their antioxidant/prooxidant activities. In our previous work (14) we showed that Antoksyd S can act as a cell proliferation modifier and growth inhibitor of murine leukemia cells (L1210). However, these results (14) cannot readily be extrapolated to the potential pharmacological applications

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Key Words: Leukemic HL-60 cells, Antoksyd S, antioxidant, prooxidant agent.

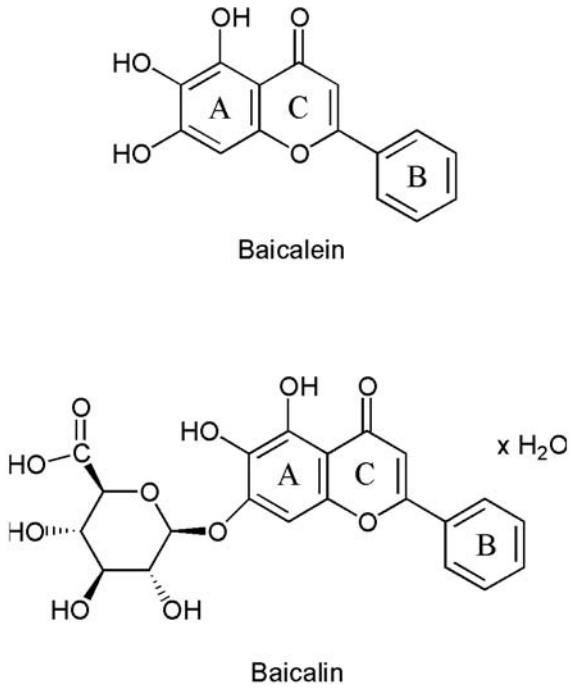

Figure 1. *Chemical structures of baicalein (BAIN) and baicalin (BA), the major chemical compounds of Antoksyd S (C/E/XXI) used in this study.*

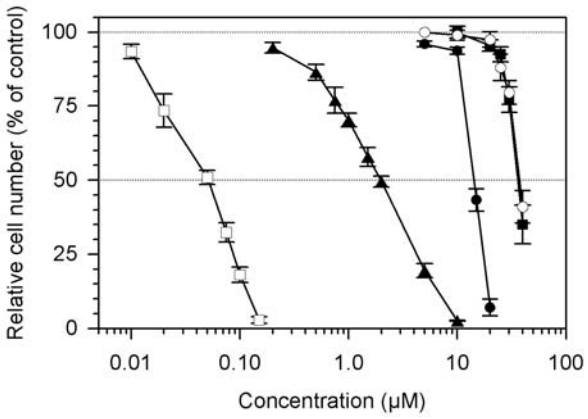

Figure 2. *The inhibitory effect of tested agents on HL-60 cell proliferation. Cells were exposed to drugs for 72 hours in growth medium. Relative cell number was estimated by a colorimetric assay as described in Materials and Methods. Data are means of 4-7 experiments ± S.D. (●) baicalein; (○) baicalin; (■) baicalin and baicalein in Antoksyd S; (□) doxorubicin; (▲) cisplatin.*

of Ant S as an antileukemic agent *in vivo*. Our objective was to investigate the effect of Ant S on human myeloid leukemia HL-60 cells as a model for human myeloid cells and to compare it with those caused by BA, BAIN as well as by the well known antileukemic drugs, doxorubicin (DOX) and cisplatin (CISP). We also present results on the relationship between the observed concentration-dependent antileukemic effects of Ant S, BA and BAIN and their antioxidative and prooxidative actions.

Materials and Methods

Chemicals. Baicalin (BA, 5,6-dihydroxy-4-oxo-2-phenyl-4H-1-benzopyran-7-yl-β-D-glucopyranosiduronic acid) and baicalein (BAIN, 5,6,7-trihydroxy-2-phenyl-4-H-1-benzopyran-4-one) (Figure 1), superoxide dismutase (SOD), xanthine oxidase (XO), lactic peroxidase (LPO), hypoxanthine (HX), dimethylsulfoxide (DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium salt (NBT), doxorubicin (DOX) and cisplatin (CISP) were purchased from Sigma-Aldrich Inc.(St.Louis, MO,USA). Calf thymus DNA (CT DNA) was also purchased from Sigma- Aldrich Inc. as sodium salt and the concentration of its solutions were determined spectrophotometrically (E_{260} = 6600 $dm^3mol^{-1}cm^{-1}$). All measurements were performed in 10 mM Tris buffer (pH 7.0). Nano-pure water from Milli Q (Millipore, USA) was used throughout. All other chemicals used were also of the highest quality commercially available. Ant S was prepared from the powdered roots of *Scutellaria baicalensis* Georgi as previously described (14) with some modifications (Patent No: P319735, Polish Office Patent, January 2004, Warsaw, Poland). The final product was obtained after crystalization as a yellow powder containing BA (80.5%), BAIN (2.58 %), wogonin (0.52 %) and its glucoside (3.21 %) (Figure 1). The purity of these components in the yellow powder (99-100 %) was proved by preparative HPLC, on Waters device with UV detector (λ = 275 nm), equipped with Millenium software and using Purosher RP-18e column (5 μM, 125 x 3.0 mm, samples – 10 μl) (Merck) and eluent acetonitrile – phosphoric acid (flow rate 1 $ml.min^{-1}$, appropriate gradients). The solutions of Ant S in DMSO were always prepared immediately before use.

Cell culture and cytotoxicity assay. The human myeloblastic leukemia cell line HL-60 was cultured in RPMI 1630 medium (Sigma-Aldrich Inc.) supplemented with 10% foetal calf serum (GIBCO) (Invitrogen Ltd.,Carlsbad, CA, USA), gentamycin (50 μg/ml) and 0.02 M HEPES buffer (GIBCO) at 37˚C in 5% CO_2 atmosphere (14).

The cytotoxic activity of the tested agents, Ant S, BA, BAIN, DOX and CISP was estimated according to the method of Carmichael *et al.* (29) with minor modification (14, 30). Briefly, HL-60 cells were seeded in 2-ml aliquots in 24-well plates at a density of 100 x 10^3 cells/ml of growth medium and exposed to drug action for 72 hours at 37˚C in triplicates. The cell number relative to the control was determined using the MTT method (29) as described previously (14). The ED_{50} value (the concentration of tested agent causing 50 % inhibition of cell growth, after 72 hours exposure) was estimated and used as a cytotoxicity index.

Interaction of BAIN with DNA. The absorption spectra of BAIN (100 μM) in 10 mM phosphate buffer (pH 7.0) were recorded in the presence of increasing amounts of CT DNA (0-3.2 mM) from 200 to 500 nm on a Cary Varian 5E spectrophotometer.

Evaluation of antioxidative and prooxidative effects in the presence of transition metal Cu(II). The superoxide ($O_2^{\bullet -}$) scavenging abilities of Ant S, BA and BAIN, respectively, were measured in a HX/XO

Table I. *Comparison of cytotoxicity of the tested flavonoids and selected anticancer drugs.*

Agent	ED_{50} (μM)*
Baicalein (BAIN)	14.4 ± 0.8
Baicalin (BA)	36.8 ± 1.1
BA and BAIN in Antoksyd S	36.5 ± 1.3
Cisplatin	1.95 ± 0.04
Doxorubicin	0.051 ± 0.006

*ED_{50} was calculated from the survival curves. Experimental conditions are described in Materials and Methods. Data are means of 5-7 experiments ± SD.

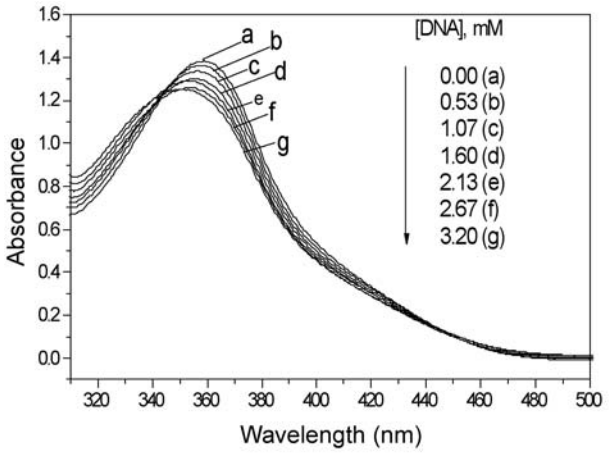

Figure 3. *The effect of increasing concentration of CT DNA (b-g) on the absorption spectrum of 100 μM BAIN (a). Experimental conditions are described in Materials and Methods.*

superoxide-generating system using NBT as a detector (31). The reaction mixtures (final volume 1 ml) contained 20 mM potassium phosphate buffer (pH 7.0), 0.5 mM HX, 1.0 mM NBT, 10 mU XO (Grade I) and the tested scavenger ranging from 5 to 100 μg, respectively. The reduction of NBT with $O_2^{\bullet -}$ was followed spectrophotometrically at 560 nm on a Cary Varian 1E spectrophotometer. The influence of Ant S, BA and BAIN on the activity of XO was tested by uric acid formation from HX in the absence (control) or in the presence of tested agents ranging from 0.2 to 50 μg. The changes of the absorbance values were measured at 295 nm, in the absence of NBT for a 15-minute incubation time.

Copper chelating capacity and reducing abilities of Ant S, BA and BAIN. These were measured spectrophotometrically using increasing concentrations of $CuSO_4$ (30-120 μM). The investigated compounds were dissolved in DMSO at a concentration of 10^{-2}M, before use. Final solutions of 100 μM BA or BAIN and Ant S (15 μg/ml) were prepared by dilution of the stock solutions in 20 mM potassium phosphate buffer (pH 7.0). After addition of the appropriate amounts of $CuSO_4$, the absorption spectra were recorded from 200 to 500 nm performing rapid scans every 20 seconds for 20 minutes. The reaction was completed in 3 minutes and the formed products were stable over the experimental time.

Results

Antileukemic activities of Ant S, BA and BAIN on HL-60 cell line. The inhibitory effects of the tested agents on HL-60 cell proliferation were investigated using the MTT test and compared with those of CISP and DOX (Figure 2). ED_{50} was determined from the curves of cell viability (CV) *vs* drug concentration (Table I). Ant S showed an obvious concentration-dependent cytotoxic effect against HL-60 cells, in the broad concentration range which was almost identical with the BA effect and about two times lower when compared with BAIN (Figure 2, Table I). Moreover, it was evident (Figure 2) that other components of Ant S, namely wogonin (0.52%) and its glucoside (3.21%), did not play a marked role in the revealed cytotoxicity.

These results correspond with the sequence of antileukemic activity determined for the five drugs acting on HL-60 cells, respectively, as follows: DOX>CISP>BAIN>Ant S, BA.

Molecular interactions of BAIN and BA with CT DNA. The absorption spectra of BAIN (100 μM) recorded in the presence of increasing amounts of CT DNA (0-3.2 mM) are shown in Figure 3. A small blue shift, hypochromism and the presence of isobestic points clearly demonstrated the formation of a complex, [BAIN-CT DNA], in the ground state. No absorption spectral changes were observed when BA was in the reaction mixtures instead of BAIN. These steady-state absorption results (Figure 3) are consistent with strong binding of BAIN to DNA and with lack of interaction of BA with DNA (not shown). Notably, it was proposed (32) that BAIN may intercalate into the DNA helix, but this remains to be confirmed.

Comparative assays of the antioxidative and prooxidative capacities of Ant S, BA and BAIN. Two different reactive species were used in the assays: superoxide ($O_2^{\bullet -}$), enzymatically generated in the HX/XO system and Cu^{2+}, a transition metal. Ant S and its major components, BA and BAIN, acted as antioxidants against superoxide (Tables II and III) but could serve as prooxidants in the presence of copper ions (Figure 4, A-C).

The HX/XO system generated superoxide ($O_2^{\bullet -}$) as measured by the reduction of NBT: this reduction was

Table II. *Effect* of Ant S, BA and BAIN on superoxide ($O_2^{\bullet-}$) generated by the XO/XH system**.*

Agent	Concentration (μg/ml)	$\Delta A_{560\ nm/min}$ (NBT reduction)	% Inhibition
Antoksyd S	0	0.1080 ± 0.0204	0
(C/E/XXI)	25.0	0.084 ± 0.009	22.0
	40.0	0.0789 ± 0.014	27.0
	50.0	0.0690 ± 0.008	36.0
	60.0	0.0504 ± 0.010	47.0
Baicalin	0	0.1248 ± 0.0114	0
(BA)	25.0	0.1153 ± 0.0196	7.9
	50.0	0.0886 ± 0.0098	29.9
	90.0	0.0520 ± 0.0081	41.6
	100.0	0.0591 ± 0.0098	47.3
Baicalein	0	0.1252 ± 0.0098	0
(BAIN)	5.0	0.0375 ± 0.0084	70.1
	8.0	0.0310 ± 0.0091	75.3
	25.0	0.0201 ± 0.0068	84.0
	50.0	0.0198 ± 0.0074	84.2
Superoxide dismutase	0	0.1252 ± 0.0098	0
(SOD)	5.0	0.0120 ± 0.0046	90.6

*The values are mean ± SD (n = 3); % Inhibition was calculated using the following formula: % $I=A_0-A_a/A_0$ x100, where A_0 denotes the extent of reduction of NBT as absorbance value at 560 nm without the tested agent and A_a-in the presence of tested agent, respectively.
** Experimental conditions are described in Materials and Methods

Table III. *Effects* of Ant S, BA and BAIN on XO activity**.*

Agent	Concentration (μg/ml)	$\Delta A_{295\ nm/min}$ (rate of urate formation)	% Inhibition
Antoksyd S	0	0.0922 ± 0.0029	
(C/E/XXI)	25.0	0.0285 ± 0.003	70.0
	50.0	0	100.0
Baicalin	0	0.0858 ± 0.0098	
(BA)	8.0	0.0595 ± 0.0084	30.7
	25.0	0.0473 ± 0.0092	45.9
Baicalein	0	0.0885 ± 0.0105	
(BAIN)	0.2	0.0722 ± 0.0084	19.5
	0.3	0.0450 ± 0.0023	49.2
	0.4	0	100.0

*The values are mean ± SD (n = 3); % Inhibition was calculated using the following formula:
$\%I=A_0-A_a/A_0$ x 100, where A_0 denotes the rate of urate formation without the tested agent and A_a- in the presence of tested agent, respectively.
** The effects were measured as rates of inhibition of HX oxidation to uric acid, catalyzed by XO as described in Materials and Methods.

inhibited by Ant S, BA and BAIN in a concentration-dependent manner (Table II). The extent of the observed inhibitory effect was investigated in the concentration range 5-100 μg/ml of the tested compound. At concentrations lower than 10 μg/ml, Ant S did not markedly affect the NBT reduction, but 60 μg/ml Ant S or 100 μg/ml BA inhibited it by about 50% (Table II). At 50 μg/ml, the tested agents inhibited NBT reduction by 36% (Ant S), 30% (BA) and 84% (BAIN). Notably, at 5 μg/ml, BAIN was only about 20% less potent a superoxide scavenger than SOD at the same concentration (Table II). As can be seen in Table III, the rates of HX oxidation to uric acid by XO were significantly changed in the presence of increasing concentrations of the investigated agents. Therefore, it is clear that Ant S, BA and BAIN are active as XO-inhibitors, with an order of activity: BAIN>Ant S>BA. These results (Table III) confirmed the previously observed XO-inhibiting activity of BA and BAIN (22) and showed, for the first time, that Ant S can act as a $O_2^{\bullet-}$ scavenger and inhibitor of XO. It worth noting that XO activity is associated with tumours and endothelial cells and that degradation processes produced a high amount of its substrate (HX) in tumours (33).

Having in mind that the $O_2^{\bullet-}$-scavenging ability (Table II) could well be correlated with the Cu^{2+}-reducing ability of flavonoids (26, 28), some experiments were carried out to determine the ability of Ant S to react with copper ions and hence to act as a prooxidant and to compare this reaction with those of BA and BAIN, respectively. As can be seen in Figure 4A, the addition of copper ions to Ant S solution caused the characteristic and stable changes (over 20 minutes) of its absorbance spectrum. The maximal absorption at 275 nm shifted immediately to 287 nm and the broad shoulder with maximum at 350 nm appeared instead of the peak at 316 nm (bathochromic shift of Band I). This result (Figure 4A) indicates that Ant S possesses copper chelating capability. The addition of peroxidase (10 mU) caused a spectral shift from 287 to 280 nm, clearly indicating the formation of H_2O_2 into the system Ant S – Cu(II)-O_2 (14, 27, 34).

Figure 4B shows the spectra of a buffered solution of BA (100 μM) scanned without or with increasing concentrations of $CuSO_4$ (30-120 μM). It can be seen that a shift of the maximal absorbance from 275 to 280 nm appeared and complete spectral changes of BA, both dependent on the copper ions concentration. The substitution at 7-position in BAIN (OH group instead of glycoside) (Figure 1) contributes to the significant differences of BA and BAIN reactions with copper (Figure 4C) compared with Ant S (Figure 4A) and BA (Figure 4B). The presence of copper ions ranging from 30 to 120 μM in BAIN solutions (100 μM)

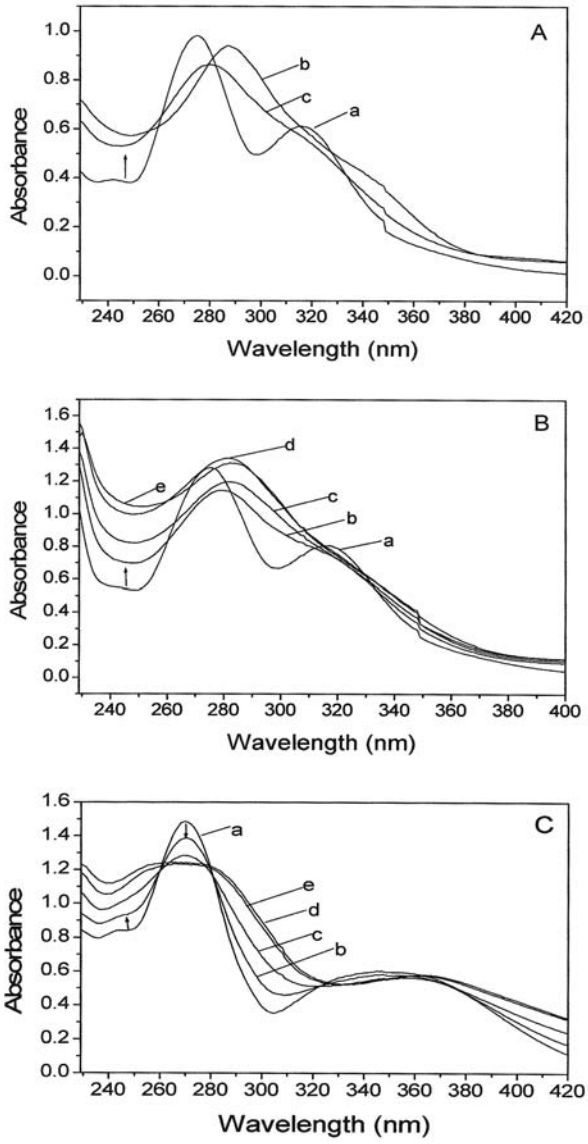

Figure 4. *Absorbance changes of Ant S(A)*, BA(B)** and BAIN(C)** in the presence of Cu(II).*
**Absorption spectrum of 15 μg/ml Ant S (a) 3 minutes after the addition of 10 μM $CuSO_4$ (b) and after addition of LPO (10 mU) (c).*
***Absorption spectra of 100 μM BA or BAIN (a), respectively, and 3 minutes after the addition of 30 (b), 60 (c), 90 (d) or 120 μM (e) $CuSO_4$, respectively. Experimental conditions are described in Materials and Methods.*

caused a copper concentration-dependent decrease of absorbance values at 270 nm and bathochromic shifts from 350 to 370 nm (Figure 4C). At lower concentrations of Cu^{2+} ions (30 or 60 μM), the clear isobestic points at 260 and 282 nm were registered. Thus, the XO-inhibiting activity of BAIN was well correlated with its Cu^{2+}-reducing ability (Table III, Figure 4C).

Discussion

The results support the aspect that the appropriate combination (ratio) of such flavonoids as BA and BAIN with known biologically active moieties (Figure 1) in the form of Antoksyd S (C/E/XXI) can efficiently exert anticancer, antioxidant and prooxidant activities.

Ant S, BA and BAIN exhibited antiproliferative activity against human leukemia cells HL-60 and they are less toxic than the commonly applied anticancer (antileukemias) drugs, CISP and DOX. Notably, the cytotoxicities of Ant S (and BA) were almost identical and it seems that the other adjuvant components, wogonin and wogonoside, do not play a role against HL-60 cells. Although *in vitro* chemosensitivity assays do not always predict the *in vivo* antileukemic activity of a drug, the therapeutic efficacy of Ant S may be much better than that of such a "strong" cytostatic as CISP or DOX, because it is non-toxic and may inhibit free radical processes at various stages of the disease.

Ant S and its constituents, BA and BAIN, may reduce the superoxide radical ($O_2^{\bullet -}$) as follows:

$$F\text{-}OH + O_2^{\bullet -} \rightarrow F\text{-}O^{\bullet} + O_2^{2-} + H^+ \quad \textbf{[1]}$$

$$O_2^{2-} + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad \textbf{[2]}$$

where F-OH represents a flavonoid structure (rings A, Figure 1) of BA, BAIN or Ant S, respectively. The mechanism of reaction **[1]** appears to involve one-electron transfer with concerted proton transfer in the transition state, as has been proposed for polyphenolic flavonoids (26, 34, 35). This reaction results in the formation of ROS (H_2O_2) as observed before (27). However, the antioxidant action of BA, BAIN and Ant S can involve another distinct mechanism: uncompetetive inhibition of $O_2^{\bullet -}$-generating XO with respect to HX as we observed in this work. Thus, it may be suggested that the high limiting – XO potency of a relatively low-toxicity agent, such as Ant S, may limit its utilization as a new anticancer agent. Notably, it was believed that BA decreased cytotoxicity with its antioxidant activity mainly based on $O_2^{\bullet -}$-scavenging ability, while BAIN acted as a good XO-inhibitor (22-26). In the present work, we showed that BA, BAIN and Ant S were all $O_2^{\bullet -}$-scavengers and XO- inhibitors with strong differences in their potency. Further investigations into these mechanisms are in progress. The observed biological effects of Ant S and its components, BA and BAIN, are believed to originate from their antioxidant activity, but the structural requirements for good antioxidant activity of flavonoids also match the essential requirements for prooxidant actions (2, 4, 7, 14, 17, 19-28). In addition to their mode of action as antioxidants, flavonoids may suppress cancer cell proliferation by inhibition of (a) enzymes other than XO involved in cellular response to growth factors or (b) of the expression of various antioxidant protein genes (36). Moreover, the molecular interaction of BAIN with DNA, observed in this work, remains to be explored in detail.

Notably, some of our preliminary experiments have shown the occurrence of DNA single- strand breaks induced in HL-60 cells by BA and BAIN (data not shown). Since BAIN has been shown to be a DNA-topoisomerase II blocker (37), its antiproliferative actions on leukemia cells (14, this work) may also be due to its interaction with DNA topoisomerases. In this study, we observed that Ant S, BA and BAIN in the presence of Cu^{2+} (without H_2O_2) can act as prooxidants rather than antioxidants and this effect increased with increasing concentration of copper ions. We suggest that the direct reactions of Ant S, BA and BAIN with copper ions could produce reactive species, as has been proposed before for other polyphenolic flavonoids-antioxidants (38, 39):

$$F\text{-}OH + 2Cu^{2+} \rightarrow Fe{=}O + 2Cu^{+} + H^{+} \quad \textbf{[3]}$$

where F-OH represents a flavonoid structure in Ant S, BA and BAIN. Moreover, it has been proposed (38, 39) that the generation of reactive Cu^{+} can cause subsequent damage of macromolecules (target), which may contribute to the following reaction sequences (39):

$$Cu^{+} + O_2 \rightarrow 2CuO \quad \textbf{[4]}$$

$$Cu^{+} + CuO_2{}^{+} + 2H^{+} \rightarrow 2Cu^{2+} + H_2O_2 \quad \textbf{[5]}$$

$$Cu^{+} + target\ [Cu^{+} - target] \quad \textbf{[6]}$$

$$[Cu^{+}\text{-}target] + H_2O_2 \rightarrow [target\text{-}Cu^{2+}] + {}^{\bullet}OH + OH^{-} \quad \textbf{[7]}$$

$$[target\text{-}Cu^{2+}] + {}^{\bullet}OH \rightarrow damaged\ target + Cu^{2+} \quad \textbf{[8]}$$

Hence, the antioxidants Ant S, BA and BAIN, which reduce Cu(II) to Cu(I), may well act as prooxidants (17, 28, 38, 39) causing the oxidative cleavage of DNA (28). It has to be emphasized here that the copper-initiated prooxidant action of Ant S and its constituents, BA and BAIN, may not be important *in vivo* where copper ions will be largely sequestered (38) and H_2O_2-metabolizing cell peroxidases will prevent the damage of targets (reactions 6-8) and the initiation of chain processes as well (2, 4, 10, 14, 40).

Thus, further investigations into the *in vivo* antioxidant and prooxidant activities of Ant S and/or BA and BAIN should be justified considering their activity against leukemia cells (14). As antileukemic compounds from medicinal remedies (41, 42) are often looked upon as alternative medicines with some hesitation or criticism (43), we investigated only chemically pure and well identified chemical compounds independently in two different laboratories in Poland. As both BA and BAIN showed antileukemic and antioxidant/prooxidant activity if applied alone, it seems reasonable to suggest further studies, also of their composite agent Antoksyd S, *in vitro* and *in vivo*.

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