NPC Natural Product Communications

2014
Vol. 9
No. 4
503 - 504

Clastogenic Effect of Atranorin, Evernic acid, and Usnic Acid on Human Lymphocytes

Gordana S. Stojanović[a*], Miroslava Stanković[b], Igor Ž. Stojanović[c], Ivan Palić[c], Vesna Milovanović[d] and Sofija Rančić[a]

[a]*Department of Chemistry, Faculty of Science and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia*
[b]*Institute of Nuclear Science Vinča, University of Belgrade, Serbia*
[c]*Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar Dr Zorana Djindjića 81, 18000 Niš, Serbia*
[d]*Technical High School, Kosančićeva 28, 37000 Kruševac, Serbia*

stgocaus@yahoo.com and gocast@pmf.ni.ac.rs

Received: December 17th, 2013; Accepted: January 19th, 2014

Three lichen secondary metabolites atranorin (**1**), evernic acid (**2**), and usnic acid (**3**), were evaluated for their *in vitro* clastogenic and antiproliferative effects on human lymphocytes using the cytochalasin-B blocked micronucleus (CBMN) assay at concentrations of 2 μg/mL, 4 μg/mL and 6 μg/mL of final culture solution. The frequency of micronucleus (MN) was scored in binucleated cells, and cytokinesis-block proliferation index (CBPI) was calculated. Among the tested compounds, **3** exhibited the most prominent effect decreasing the frequency of MN in the range of 42.5% - 48.9%, that is about double of the positive control amifostin WR-2721 that reduces MN frequency for 22.0%. The effect of evernic acid was approximately equal to action of amifostin (23.2% -32.9%). Atranorin at concentrations of 2 μg/mL and 4 μg/mL decreasing the frequency of MN only for 11.1% and 1.8%, while in concentration of 6 μg/mL increases the frequency of MN for 9.6 %. The comparable CBPI values of the investigated compounds and control suggested that they did not show a statistically significant inhibitory effect on lymphocyte cell proliferation at applied concentrations.

Keywords: Atranorin, Evernic acid, Usnic acid, Lichen secondary metabolites, Human lymphocytes, Micronucleus test.

Environmental mutagens can induce the formation of micronuclei in the cytoplasm of interphase cells. Micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. Micronucleus expression in peripheral blood lymphocytes is well established as a method to monitor chromosome damage in human population. The cytokinesis-block micronucleus technique enables micronuclei to be specifically scored in cells that have completed nuclear division and is, therefore, not influenced by variations in cell division kinetics, and it has been shown to be a sensitive and reliable index of chromosome damage [1].

Continuing our investigation on the biological activities of the lichen substances [2, 3], herein we report the clastogenic effect (micronucleus distribution) and antiproliferative effect (CBPI) in peripheral blood lymphocytes of the atranorin (isolated from *Hypogymnia physodes*), evernic acid (isolated from *Evernia prunastri*), and (+)-usnic acid (isolated from *Usnea barbata*). The structures of examined compounds **1**, **2**, and **3**, as well as structures of positive control (amifostine) and negative control (mitomycin C, MMC) are given on Figure 1. The lichen secondary metabolites **1-3** were tested for *in vitro* protective effect on chromosome aberrations in peripheral human lymphocytes using the cytochalasin-B blocked micronucleus (CBMN) assay in doses of 2.0, 4.0, and 6.0 μg per mL of final culture solution. The frequency of MN was scored in binucleated cells, and cytokinesis-block proliferation index CBPI were calculated. Amifostine (radioprotectant, previously known as WR-2721) was used as positive control at concentration of 1.0 μg per mL. Mitomycin C, a clastogenic agent that has been used to study the susceptibility of cell to chromosomal damage and cytotoxic effects [4] was used as negative control. The results are shown in Table 1.

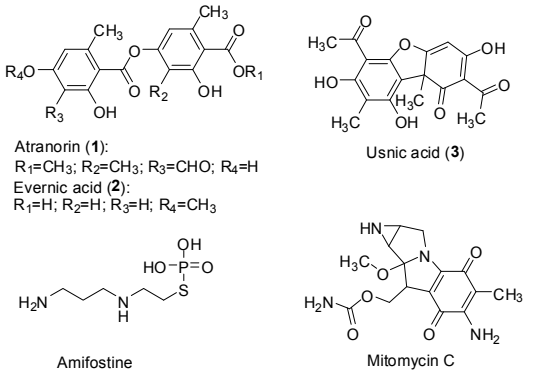

Figure 1: The structures of atranorin (**1**), evernic acid (**2**), usnic acid (**3**), amifostine, and mitomycin C.

From the presented results could be observed following:

- Higher concentrations of compounds under the study causes smaller decrease in the frequency of micronuclei. Atranorin at a concentration of 6 mg per mL, even increasing the frequency of MN for 9.6% compared to the control.
- Among the tested compounds usnic acid exhibited the most prominent effect on decreasing frequency of MN (48.9% for 2 μg/mL, 46.4% for 4 μg/mL and 42,5% for 6 μg/mL compared with the control cell cultures, $p < 0.01$ for all concentrations), following by evernic acid (32.9% for 2 μg/mL, 26.8% for 4 μg/mL and 23.2% for 6 μg/mL compared with the control cell cultures, $p < 0.01$ for all concentrations), and atranorin (11.1% for 2 μg/mL, 1.8% for 4 μg/mL.
- The cell cultures treated with amifostine WR-2721 (positive control) at concentration of 1 μg/mL gave a significant ($P < 0.01$) decrease in the MN frequency of 22.5% comparing to control cell cultures. Comparing the effects of amifostine

Table 1: Incidence of MN, CBPI, and frequency of MN in cell cultures of human lymphocytes treated with amifostine, mitomycin C and different concentration of lichen secondary metabolites **1**, **2**, and **3**.

Sample	µg/mL	µmol/mL	MN/1000 Bn cell	% Bn cell with MN	MN/Bn cell	CBPI	Frequency of MN (%)
Control	0.0	0.0	28.0 ± 0.8	2.3 ± 0.1	1.1 ± 0.0	1.7 ± 0.0	100
Amifostine	1.0	$4.6x10^{-3}$	21.7 ± 1.1[a,]	1.7 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	77.5
Mitomycin C	0.2	$5.9x10^{-4}$	37.8 ± 2.9[a*,b,]	3.3 ± 0.2	1.1 ± 0.0	1.7 ± 0.0	135.0
Atranorin (**1**)	2.0	$5.3x10^{-3}$	24.9 ± 0.9[a*,c]	2.1 ± 0.1	1.2 ± 0.1	1.6 ± 0.0	88.9
	4.0	$10.6x10^{-3}$	27.5 ± 1.0[b,c*]	2.3 ± 0.1	1.2 ± 0.0	1.6 ± 0.0	98.2
	6.0	$16.0x10^{-3}$	30.7 ± 2.0[b]	2.6 ± 0.2	1.2 ± 0.0	1.6 ± 0.0	109.6
Evernic acid (**2**)	2.0	$6.0x10^{-3}$	18.8 ± 0.6[a,c]	1.6 ± 0.0	1.1 ± 0.0	1.6 ± 0.0	67.1
	4.0	$12.0x10^{-3}$	20.5 ± 0.4[a,c]	1.8 ± 0.0	1.1 ± 0.0	1.6 ± 0.0	73.2
	6.0	$18.0x10^{-3}$	21.5 ± 0.7[a,c]	1.8 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	76.8
Usnic acid (**3**)	2.0	$5.8x10^{-3}$	14.3 ± 1.4[a,b,c]	1.2 ± 0.1	1.2 ± 0.0	1.7 ± 0.1	51.1
	4.0	$11.6x10^{-3}$	15.0 ± 1.3[a,b,c]	1.2 ± 0.2	1.3 ± 0.1	1.7 ± 0.1	53.6
	6.0	$17.4x10^{-3}$	16.1 ± 1.9[a,b*,c]	1.3 ± 0.2	1.3 ± 0.1	1.7 ± 0.1	57.5

Data represent as means±SD (standard deviation) of three experimental determinations.
MN/1000 Bn cells - incidence of micronuclei in 1000 binucleated cells. % Bn cells with micronuclei. MN/Bn cells - incidence of micronuclei in binucleated cells.
CBPI- cytokinesis-block proliferation index.
a Compared with control groups, statistically significant difference $p < 0.01$.
a* Compared with control groups, statistically significant difference $p < 0.05$.
b Compared with amifostine – WR 2721, statistically significant difference $p < 0.01$.
b* Compared with amifostine – WR 2721, statistically significant difference $p < 0.05$.
c Compared with mitomycin C, statistically significant difference $p < 0.01$.
c* Compared with mitomycin C, statistically significant difference $p < 0.05$.

(1 µg/mL) and examined compounds, usnic acid (2 and 4 µg/mL) demonstrated statistically significant difference in the decreasing frequency of MN. Roughly, its activity was about two times higher than the activity of amifostine.

- The treatment with alkylating agent MMC (negative control) at concentration of 0.2 µg/mL gave a significant ($P < 0.05$) increase in the MN frequency of 35% comparing to control cell cultures. Compared to the MMC, all of the test substances at all concentrations showed a statistically significant reduction in the frequency of MN except for atranorin at a concentration of 6 µg/mL.
- The CBPI values of the investigated samples, amifostine, mitomycin C and control suggested that they did not show a statistically significant effect on lymphocyte cell proliferation at the applied concentrations. It is important since MN expression is dependent on cell division.

Our previous paper [3] showed that lichen depsidones, physodalic acid, physodic acid, and 3-hydroxy physodic acid, also decreased the frequency of MN at a concentrations of 1.0 and 2.0 µg/mL more than amifostine at a concentration of 1 µg/mL. However, their effects were smaller than the effect of usnic acid. Comparing the compound structures and decreasing the frequency of MN it seems that rigid structure of dibenzofuran (usnic acid) is more effective than structures of depsidones (physodalic acid, physodic acid, and 3-hydorxy physodic acid), and depsides (atranorin and evernic acid). Furthermore, introduction of methyl group instead of hydrogen into carboxyl and hydroxyl groups (atranorin versus evernic acid) reduces the decreasing frequency of MN.

In summary, this study showed that lichen secondary metabolites **1**, **2**, and **3** reduced the frequency of MN in concentration dependent manner, and the lowest applied concentration of 2.0 µg/mL being the most effective. Among examined compounds dibenzofuran derivative usnic acid was (**3**) more active than atranorin, and evernic acid. Usnic acid at a concentration of 2 µg/mL decreased MN frequency for 48.9%, while in the same experiment amifostine at concentration of 1 µg/mL decreasing the MN frequency for 22.5%. *E*xpressed activity in preventing damage and/or enhancing DNA repair could be added to a long list of usnic acid bioactivity [5, 6].

Experimental

General: Atranorin (**1**) was isolated from *Hypogymnia physodes* [8], evernic acid (**2**) isolated from *Evernia prunastri* [9] and (+)-usnic acid (**3**) isolated from *Usnea barbata* [10]. The purity of the isolated compounds was determined by HPLC-DAD and amounted 99.8, 98.6, and 98.5 % for **1**, **2**, and **3**, respectively.

Cytokinesis-block micronucleus (CBMN) assay: This test was performed as previously described [7]. The cell culture lymphocytes were treated with 2.0, 4.0 and 6 µg/mL of the compounds **1-3.** Amifostine WR-2721 (98% S-2[3-aminopropylamino]-ethyl-phosphothioic acid; Marligen-Biosciences) at 1 µg/ml) was used as a positive control, and mitomycin C (MMC, Sigma-ALDRICH) (0.2 µg/mL, in phosphate buffer) as a negative control. Three experiments (with three replications) were performed for each sample. The results are expressed as the means ± SD.

Statistical analysis: The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at $p< 0.01$ and $p<0.05$.

Acknowledgments - Financial support of the Ministry of Education, Science and Technological Development of Serbia (Project No. 172047) is gratefully acknowledged.

References

[1] Fenech M, Morley AA. (**1993**) The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutation Research*, ***285***, 35-44.
[2] Pavlovic V, Stojanovic I, Jadranin M, Vajs V, Djordjević I, Smelcerovic A, Stojanovic G. (**2013**) Effect of four lichen acids isolated from *Hypogymnia physodes* on viability of rat thymocytes, *Food and Chemical Toxicology*, ***51***, 160-164.
[3] Stojanović I, Stanković M, Jovanović O, Petrović G, Šmelcerović A, Stojanović G. (**2013**) Effect of *Hypogymnia physodes* extracts and their depsidones on micronucleus distribution in human lymphocytes. *Natural Product Communications*, ***8***, 109-112.
[4] Roncada T, Vicentini VEP, Mantovani MS. (**2004**) Possible modulating actions of plant extracts on the chromosome breaking activity of MMC and Ara-C in human lymphocytes *in vitro*. *Toxicology in Vitro*, ***18***, 617–622.
[5] Ingolfsdottir, K. (**2002**) Usnic acid. *Phytochemistr*y, ***61***, 729-736.
[6] Cocchietto M, Skert N., Nimis P, Sava G. (**2002**) A review on usnic acid, an interesting natural compound. *Naturwissenschaften*, ***89***, 137-146.
[7] Aljancić I, Stanković M, Tesević V, Vujisić L, Vajs V, Milosavljević S. (**2010**) Protective effect on human lymphocytes of some flavonoids isolated from two *Achillea* species. *Natural Product Communications*, ***5***, 729-732.
[8] Lukač M, Onderka M. (**2006**) Applicaton of DDQ (2,3-dichloro-5,6-dicyano-benzoquinone) in detection of lichen acids. *Acta Facultatis Pharmaceuticae Universitatis Comenianae*, ***53***, 160-166.
[9] Kosanić M, Manojlović N, Janković S, Stanojković T, Ranković B. (**2013**) *Evernia prunastri* and *Pseudoevernia furfuraceae* lichens and their major metabolites as antioxidant, antimicrobial and anticancer agents. *Food and Chemical Toxicology*, ***53***, 112-118.
[10] Tosun F, Akyüz Kizilay Ç, Sener B, Vural M. (**2005**) The evaluation of plants from turkey for *in vitro*. Antimycobacterial activity. *Pharmaceutical Biology*, ***43***, 58-63.