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Photoactivated hypericin is not genotoxic

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Abstract. The study was designed to test the potential photogenotoxicity of hypericin (HYP) at three different levels: primary DNA damages, gene mutations and chromosome aberrations. Primary genetic changes were detected using the comet assay. The potential mutagenic activity of HYP was assessed using the Ames/*Salmonella typhimurium* assay. Finally, the ability of photoactivated HYP to induce chromosome aberrations was evaluated by the *in vitro* mammalian chromosome aberration test and compared to that of non-photoactivated HYP. The results have shown that photoactivated HYP can only induce primary DNA damages (single-strand DNA breaks), acting in a dose-response manner. This activity depended both on HYP concentrations and an intensity of the light energy needed for its photoactivation. However, mutagenic effect of photoactivated HYP evaluated in the Ames assay using three bacterial strains *S. typhimurium* (TA97, TA98 and TA100) was not confirmed. Moreover, photoactivated HYP in the range of concentrations (0.005–0.01 μg/ml) was not found to be clastogenic against HepG2 cells. Our findings from both the Ames assay and the chromosome aberrations test provide evidence that photoactivated HYP is not genotoxic, which might be of great importance mainly in terms of its use in the photodynamic therapy.

Key words: Hypericin — Photogenotoxicity — Comet assay — Ames/*Salmonella typhimurium* assay — *In vitro* mamalian chromosomal aberration test

Introduction

Hypericin (HYP) is one of the most important bioactive compounds extracted from *Hypericum perforatum* L. It is known mainly for its antibacterial activity as it inhibits the growth of *Staphylococcus aureus* and *S. epidermidis* (Feyzioğlu et al. 2013). Hypericin has also an antiviral activity inactivating murine cytomegalovirus (MCMV), Sindbis virus, and human immunodeficiency virus type 1 (HIV-1) (Hudson et al. 1991).

HYP has been considered to be one of the most powerful photosensitive substances found in nature (Castano et al. 2004). As an effective photosensitizer it is used in the photodynamic therapy (PDT), and is responsible for antitumor and antiproliferative properties of *Hypericum perforatum* L. extract (Karioti and Bilia 2010). Koval et al. (2010) demonstrated the antiproliferative ability of HYP showing that hypericin-mediated photodynamic therapy (HY-PDT) was able to degrade human epidermal growth factor receptor 2 that may be responsible for the resistance of breast cancer to therapeutics (Karioti and Bilia 2010; Solár et al. 2011). PDT has become a promising option for cancer treatment (Ahn et al. 2014). PDT is a clinically approved and minimally invasive procedure that can exert a selective cytotoxic activity towards malignant cells. The procedure involves administration of photosensitizing agent, which is subsequently exposed to a light source of suitable wavelength and can react through free radical mechanisms (Castano et al. 2004; Solár et al. 2011).

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HYP can be activated by light in the range of wavelengths between 300–700 nm that includes visible light (400–700 nm) as well as UVA radiation (320–400 nm) (Fox et al. 1998; Jendželovská et al. 2014). In the presence of oxygen, a series of events leads to a direct tumor death, damage to the microvasculature, and induction of a local inflammatory reaction (Garg et al. 2012). The recent interest in HY-PDT results from its effective selective tumors-localizing properties (Vandenbogaerde et al. 1998; Agostinis et al. 2002; Miskovsky 2002; Head et al. 2006). However, Traynor et al. (2005) showed that although the combination of HYP and UVA light increased the genotoxic burden, when all factors are taken into account, the risk of significant photogenotoxic damage incurred by the combination of *Hypericum* extracts and UVA phototherapy may be low in majority of individuals.

Jendželovský et al. (2009) and Jendželovská et al. (2014) proved that HYP is able to reduce the action of a wide spectrum of antineoplastic agents by increasing the expression levels of two ABC transporters (MRP1 and BCRP) in the adenocarcinoma cell line.

Our study is based on our previous research aimed at the evaluation of the potential genotoxic and antigenotoxic activities of non-photoactivated HYP (Miadokova et al. 2010). This work was focused on a detection of potential genotoxic effects of photoactivated HYP at the levels of primary DNA damage, gene mutations and chromosome aberrations using three different genetic test systems.

Materials and Methods

Tested compound – hypericin (HYP)

Chemically, HYP belongs to naphthodianthrones. HPLC grade (98%) HYP (4,5,7,4',5',7'-hexahydroxy-2,2'-dimethylnaphtodiantrone) (AppliChem, Germany) was prepared as a stock solution (10 mg/ml) in DMSO and subsequently diluted to required concentrations.

Experimental cells and cell lines

Lymphocytes used to monitor potential genotoxic effects of photoactivated HYP in the comet assay were isolated from peripheral blood obtained by *finger prick* method. 40 µl of peripheral blood was pipetted into 1 ml of ice-cold 1×PBS and allowed to stand on ice for 30 min. Lymphocytes were separated from whole blood samples by the standard centrifugation with 100 µl of the Histopaque medium (Sigma – Aldrich). After the centrifugation, 200 µl of isolated cells were resuspended in 1 ml of 1×PBS buffer and re-centrifuged. The lymphocytes in pellets were collected and used for experiments.

A set of three *Salmonella typhimurium* tester strains was used for mutagenicity testing (Ames assay). *S. typhimurium strains* TA97, TA98 and TA100 were obtained from the Czech Collection of Microorganisms (Brno, the Czech Republic).

The cell line HepG2, derived from human hepatocarcinoma, used for the chromosome aberrations evaluation was established at the Wistar Institute of Anatomy and Biology, Philadelphia, PA, USA. The cell line was provided by A. Collins (Department of Nutrition, University of Oslo, Norway). The cells were cultured in Williams medium (PAN-Biotech GmbH, Germany) with 10% fetal bovine serum (PAN-Biotech GmbH, Germany). The Williams medium was supplemented with gentamicin (50 µg/ml) (Sandoz, Slovenia). The cells were cultured in plastic Petri dishes in CO_2/air (5%: 95%) environment at 37°C, as previously described by Miadokova et al. (2010).

Photoactivation

Photoactivation was carried out according to the procedure previously described by Šemeláková et al. (2012). In brief, samples were placed on a diffuser glass of a specially modified lamp. The irradiating device consisted of eleven L18W/30 fluorescent tubes (Osram, Berlin, Germany) with the maximum emission range from 530 to 620 nm. Each sample was exposed to irradiation for 10, 15 or 25 min, which was an equivalent to energy doses of 1.46 J/cm^2; 2.19 J/cm^2 and 3.65 J/cm^2, respectively. The temperature did not exceed 37°C.

The alkaline comet assay

The comet assay (Single Cell Gel Electrophoresis) was performed according to (Horváthová et al. 1999; Collins, 2004; Gafrikova et al. 2014). Briefly: Blood samples collected by *finger prick* were put into 1×PBS and left on ice for 30 min. Lymphocytes were isolated as described above. The pellet of cells was re-suspended in 1% LMP (low melting point) agarose. A volume of 100 µl of lymphocytes/cells was mixed with 1% NMP (normal melting point) agarose. Cover slips were placed on the top of the slides, which solidified at 4°C. After removing the cover slips, the cells were treated with different concentrations of HYP (0.05–0.75 µg/ml) prepared in PBS solution and cultivated for 60 min at 37°C. As a positive control, 100 µmol/l hydrogen peroxide (H_2O_2) was used. For a negative control, the cells were left untreated, in fresh 1×PBS solution for 1 h at 37°C. In case of photoactivated samples, after the incubation with HYP, the cells were washed with fresh PBS and placed on the diffuser glass of a specially modified lamp for 10, 15 or 25 min, which is an equivalent to doses of energy of 1.46 J/cm^2, 2.19 J/cm^2 or 3.65 J/cm^2, respectively. Cells were then lysed

by immersing slides into the lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris-HCl, pH 10 and 1% Triton X-100) at 4°C for 1 h. After the lysis, the slides were transferred into an electrophoretic tank containing fresh and chilled alkaline electrophoretic solution (300 mmol/l NaOH, 1 mmol/l Na_2EDTA, pH 13), and left for 20 min at 4°C to allow the DNA to unwind. The electrophoresis was run for 30 min at 4°C at 25 V and 260–320 mA. The slides were neutralized by a single wash in ice cold 1×PBS for 5 min followed by one wash in cold dH_2O for another 5 min. Finally, slides were stained with ethidium bromide (20 μg/ml), and in each sample 100 random nucleoids were scored at magnification 400× using a fluorescence microscope OLYMPUS BX 51. The DNA damage was evaluated using the Comet visual computer software. The evaluation depended on the relative intensity of DNA fluorescence in the comet tail.

Ames/Salmonella typhimurium assay

Ames assay was conducted according to the method revised by Maron and Ames (1983). To identify potential mutagenic effect of photoactivated HYP, three tester strains of *Salmonella typhimurium* were used – TA97, TA98 and TA100.

The purpose of the assay was to determine the possible mutagenic potential of photoactivated HYP (20–100 μg/plate).

As a positive control, the direct mutagen 9-aminoacridine (9-AA; 50 μg/plate) was used for strain TA97, 4-nitroquinoline-N-oxide (4NQO; 20 μg/plate) for strain TA98 and sodium azide (NaN_3, 50 μg/plate) for strain TA100.

The assay was carried out in test tubes containing 0.1 ml (approximately 1×10^8 cells/ml) of overnight bacterial culture, cultivated in 50 ml of LB Broth (Lennox) medium and 2.5 ml of top-agar. Then the tested HYP (20, 50 or 100 μg/plate) was supplemented.

After the addition of HYP to the bacterial culture, the half of the test tubes was exposed to a light dose of 3.65 J/cm^2 (using a lamp specially modified for these experiments) for 30 min at 37°C, following by additional incubation for 30 min at 37°C. The half of the test tubes which was not photoactivated was also incubated for 60 min at 37°C. Then the top agar was supplemented and the contents were plated on minimal medium plates. After 5 days of incubation at 37°C, *his*$^+$ revertants were counted.

In vitro mammalian chromosome aberration test

The experiments were based on the OECD (Organization for Economic Cooperation and Development) recommendations, test No. 473: *In vitro* mammalian chromosome aberration test (Galloway et al. 1994). The HepG2 cells were seeded into Petri dishes (Ø 60 mm; inoculum 1×10^6 cells/dish) and allowed to grow for 24 h. Then the cultures were treated with different concentrations of HYP (0.005–1.0 μg/ml) added into the media. After the further 16 h, the initial medium was replaced by a fresh medium, and simultaneously, a half of the samples was photoactivated using a specially modified lamp for 25 min (3.65 J/cm^2) in the presence of oxygen. The other half of the samples was not photoactivated. The cells were then cultivated for additional 36 h. As a positive control, cisplatin (1 μg/ml) was used. Colchicin had been added into the media 3 h before the karyological preparations were processed. Slides were stained with 2% aqueous Giemsa-Romanowski solution for 10 minutes. Afterwards, preparations were air-dried.

Chromosomal aberrations in cells were evaluated by a microscopic examination. For each sample, 100 metaphases were analysed, if it was possible. We focused on the following structural aberrations: breaks-chromatid, iso-chromatid, exchanges-dicentrics, rings, tri-radials and quadri-radials. Some gaps were observed as well, but those were not included in the total number of chromosome aberrations, according to the OECD recommendations (Galloway et al. 1994). The number of aberrant metaphases and the total number of chromosomal aberrations was statistically evaluated.

Statistical analysis

Each experiment was done in triplicate and statistical analysis was done by the Student's *t*-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The results of cytogenetic assay were statistically evaluated using the test of the difference of two relative values.

Results

The ability of non-photoactivated and photoactivated HYP (0.05–0.75 μg/ml) to induce single-strand DNA breaks (SSBs) in human lymphocytes was evaluated by the alkaline comet assay. Photoactivated HYP, in contrast to non-photoactivated HYP, exerted the ability to induce primary DNA damages in a dose-dependent manner (Figure 1). Percentage of damaged DNA in each sample treated with photoactivated HYP was significantly increased in comparison to the negative control. Moreover, the effect of selected concentrations of HYP (0.05–0.75 μg/ml) depended on the intensity of the light energy, also in the dose-response manner (Figure 2).

The effects of both non-photoactivated and photoactivated HYP (20–100 μg/plate) on the bacterial strains *S. typhimurium* TA97, TA98 and TA100 were evaluated using the Ames/*Salmonella typhimurium* assay. Both non-photoactivated and photoactivated HYP were not mutagenic (Table 1).

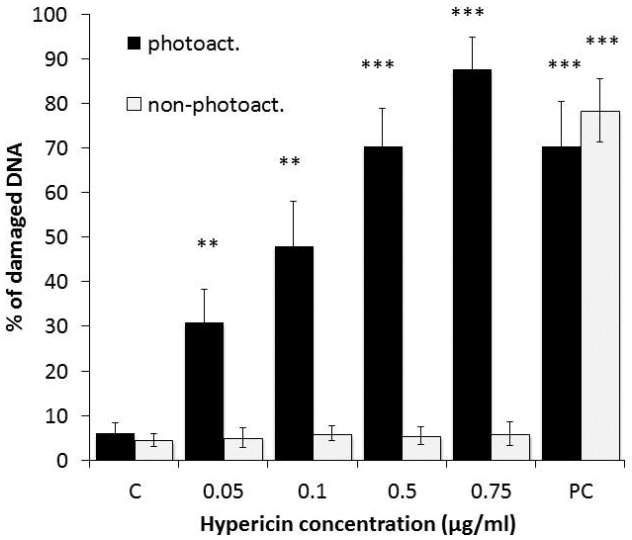

Figure 1. Potential genotoxic activity of different concentrations of photoactivated and non-photoactivated hypericin on human lymphocytes determined by the comet assay. Human lymphocytes were treated with different concentrations of hypericin (0.05–0.75 μg/ml) and cultivated for 60 min at 37°C. Statistical significance was considered for p values as follows: ** $p < 0.01$ and *** $p < 0.001$. C, negative control – cells without treatment with hypericin; PC, positive control – cells treated with 100 μmol/l hydrogen peroxide.

The standard cytogenetic assay was used to detect the ability of non-photoactivated and photoactivated HYP to induce double-strand breaks (DSBs) that are crucial for the formation of chromosomal aberrations. As it is presented in Table 2, when comparing the intact control (IC) with the solvent one (SC), we did not detect any significant difference. Therefore DMSO can be considered as an acceptable solvent. After that we compared all the samples treated with HYP against the solvent control (SC). There were no significant differences in two main cytogenetic parameters – the number of aberrant metaphases and the total number of chromosome aberrations in cells treated with either photoactivated or non-photoactivated HYP in comparison to the SC. Thus, neither non-photoactivated nor photoactivated HYP exhibited clastogenic effects.

After treating HepG2 cells with photoactivated HYP at the concentration of 0.05 μg/ml, only 32 evaluable mitoses were recorded. No accessible mitosis were detected in HepG2 samples treated with higher concentrations of photoactivated HYP (0.1–1.0 μg/ml) due to the inhibiton of cell proliferation by photoactivated HYP (data not shown).

Discussion

During the last decades PDT has been established as a powerful alternative by health agencies in several countries approved for the treatment of various malignant and some non-malignant diseases. However, its potential genotoxic activity has not yet been established properly. Therefore, we have decided to complement this gap of knowledge in regard to the potential genotoxicity of photoactivated HYP. We studied the potential genotoxic effects of photoactivated HYP at the level of the primary DNA damages, at the level of gene mutations and chromosome aberrations using three different test systems. The alkaline comet assay is a rapid and sensitive

Table 1. Ames assay (*Salmonella typhimurium*)

	Concentration (μg/plate)	Frequency of his^+ revertants
Strain TA97		
NC	0	110 ± 12
HYP	20	112 ± 12
HYP	50	102 ± 17
HYP	100	100 ± 6
$_{ph}$NC	0	105 ± 12
$_{ph}$HYP	20	103 ± 13
$_{ph}$HYP	50	93 ± 14
$_{ph}$HYP	100	90 ± 16
9-AA	50	906 ± 124*
Strain TA98		
NC	0	51 ± 11
HYP	20	36 ± 6
HYP	50	31 ± 10
HYP	100	30 ± 11
$_{ph}$NC	0	51 ± 9
$_{ph}$HYP	20	48 ± 10
$_{ph}$HYP	50	46 ± 10
$_{ph}$HYP	100	45 ± 9
4-NQO	20	363 ± 33**
Strain TA100		
NC	0	146 ± 20
HYP	20	119 ± 12
HYP	50	123 ± 11
HYP	100	133 ± 10
$_{ph}$NC	0	128 ± 16
$_{ph}$HYP	20	147 ± 10
$_{ph}$HYP	50	147 ± 10
$_{ph}$HYP	100	146 ± 10
NaN_3	50	1809 ± 41**

Potential mutagenic activity of non-photoactivated (HYP) or photoactivated ($_{ph}$HYP) hypericin tested on *S. typhimurium* using the Ames test. NC, negative control; 9-AA, 9-aminoacridine (50 μg/plate) used as a positive control for the strain TA97; 4-NQO, 4-nitroquinoline-N-oxide (50 μg/plate) used as a positive control for the strain TA98; NaN_3, sodium azide (25 μg/plate) used as a positive control for the strain TA100. Each value represents the mean ±SD of three separate experiments. * $p \leq 0.01$; ** $p \leq 0.001$.

fluorescent microscopic method (Firouzi et al. 2015). The comet assay on lymphocytes revealed that photoactivated HYP induced primary DNA damages represented by SSBs, which are considered as transient promutagenic lesions indicating potential mutagenic effects of DNA damaging agents. Possibly, they could be related to apurinic/apyrimidinic sites (alkali-labile sites appearing as DNA breaks) and also represent intermediates in cellular repair, since NER (nucleotide excision repair) and BER (base excision repair) cut out the damage and replace it with undamaged nucleotides (Collins et al. 2008). The percentage of DNA damage depended on two essential components: HYP concentration and an activation energy, both in a dose-response manner as we showed in Figure 2 using human lymphocytes. Traynor et al. (2005) used HaCaT cell line treated with HYP activated by UVA (either 4 or 0.4 J/cm^2). As for activation of HYP in PDT the visible light is recently used (Jendželovský et al. 2009; Jendželovská et al. 2014), in our experiment we used lymphocytes treated with HYP activated by visible light (1.46–3.65 J/cm^2). Our results are consistent with those previously reported by Traynor et al. (2005) showing that

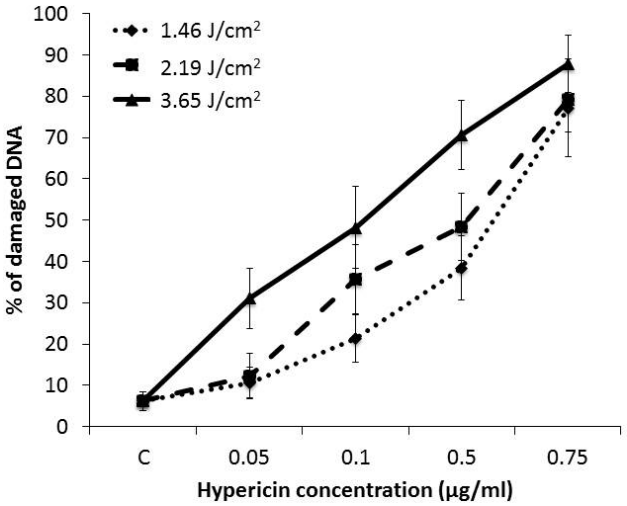

Figure 2. The percentage of DNA damage induced with hypericin activated by different doses of light energy (1.46 J/cm^2, 2.19 J/cm^2 and 3.65 J/cm^2) in human lymphocytes determined by the comet assay. C, negative control – cells without the treatment with hypericin.

Table 2. Potential clastogenic effect of non-photoactivated and photoactivated hypericin

HYP/PC CisPt (μg/ml)	Total number of metaphases	Number of aberrant metaphases	Number of chromosome aberrations									Total number of CA
			Chromatid		Isochromatid		Exchange					
			g	b/f	g	b/f	ring	dic	qr	tr	dmin	
IC	100	7	–	2	1	1	–	5	–	–	–	8
SC	100	9	–	3	–	–	–	7	–	–	–	10
PC CisPt 1.0	100	50***	13	64	2	6	–	–	2	3	–	75***
HYP 0.005	100	7	–	6	–	–	–	1	–	–	–	7
HYP 0.01	100	9	–	6	1	–	–	3	–	–	–	9
HYP 0.05	100	7	–	6	–	–	1	–	–	–	–	7
HYP 0.1	100	5	–	2	–	–	–	–	–	–	–	5
HYP 0.5	100	7	1	3	1	–	–	4	–	–	–	7
HYP 0.75	100	5	–	2	–	–	–	3	–	–	–	5
HYP 1	100	8	–	4	1	–	–	4	–	–	–	8
$_{ph}$IC	100	7	–	2	1	–	–	5	–	–	–	7
$_{ph}$SC	100	6	1	5	–	–	–	2	–	–	–	7
$_{ph}$HYP 0.005	100	4	1	2	–	–	–	2	–	–	–	4
$_{ph}$HYP 0.01	100	6	1	4	–	–	1	1	–	1	–	7
$_{ph}$HYP 0.05	32	1	–	1	1	–	–	–	–	–	–	1

A total of 100 metaphases were scored in each sample. The number of chromatid and isochromatid gaps was recorded for each treatment group, however, they were not included in the overall assessment of chromosome damage. HYP, hypericin; CisPt, cisplatin; PC, positive control; IC, intact control; SC, solvent control (DMSO); CA, chromosome aberrations; g, gap; b/f, break and/or fragment; ring, ring (circular) chromosome; dic, dicentric; qr, quadriradial; tr, triradial; dmin, double minutes. Significant difference in comparison with the solvent control (SC) is marked with asterisks: *** $p < 0.001$. Photoactivated samples are marked with symbol ($_{ph}$). No accessible mitosis were detected in samples treated with the highest concentrations of photoactivated HYP (0.1; 0.5; 0.75; 1.0 μg/ml).

both UVA and visible light similarly activate HYP in a dose-response manner.

As two major genetic alternations – gene mutations and chromosomal aberrations are often implicated in the activation of oncogens or inactivation of tumor suppressor genes and thus are important in the malignant transformation process (Khan et al. 1995), we tried to find out whether photoactivated HYP could induce such DNA lesions.

In the course of photodynamic therapy, photosensitizer passes from the ground (singlet) state to the excited singlet state after absorption of photons. At this stage, it can undergo a process called intersystem crossing (Castano et al. 2004), whereby the spin of the excited electron inverts to form the excited triplet-state. Subsequently, two types of reactions might take place; type I reaction, during which the photosensitizer reacts directly with the substrate – a cell membrane or a molecule, and type II reaction, where the photosensitizer in triplet state transfers energy directly to molecular oxygen to form the excited singlet oxygen forms. Both types trigger further reactions, which result in the production of ROS that are toxic to many cellular structures and macromolecules (DNA, lipids, enzymes) (Castano et al. 2004; Karioti and Bilia 2010; Čavarga et al. 2014).

Mikeš et al. (2009) previously reported that PDT depended on the type of the photosensitizer used, its intracellular localization, as well as the genetic and metabolic potential of the monitored cell line. In our experiments, we used human lymphocytes (comet assay) and HepG2 cell line (chromosome aberration test). Our results confirmed and complemented our previous conclusions that non-photoactivated HYP did not exert genotoxic activity in the bacterial, yeast and algal assays used (Miadoková et al. 2010). Similar results were obtained by Gyenge et al. (2012) on UMB-SCC 745 and UMB-SCC 969 cell lines derived from oropharynx and tonsils head and neck squamous cell carcinomas, respectively. Kersten et al. (1999) found out that HYP was slightly photogenotoxic only at the highest concentration used. Similar concetrations were cytotoxic in our experiments (Table 2). Moreover, they used the micronucleus assay and different cell line (V79).

The standard *S. typhimurium* tester strains (TA97, TA98, TA100) used in the Ames assay contain mutations in histidine operon. Strain TA100 enables to detect mutagens inducing base-pair substitutions, strain TA97 frameshift mutations and strain TA98 allows to detect base-pair substitutions and frameshift mutations (Maron and Ames 1983).

The Ames assay with non-photoactivated HYP of unknown purity was firstly performed by Turek et al. (1997) with *S. typhimurium* strains TA98 and TA100. They found out that non-photoactivated HYP was not mutagenic. In our previous paper (Miadokova et al. 2010) we have shown that none of the concentrations of non-photoactivated HYP exerted mutagenic effects on strain TA97. Now we present the results of the Ames/*Salmonella typhimurium* assay on 3 bacterial strains (TA 97, TA 98 and TA100). We revealed that HYP was not mutagenic either in non-photoactivated or photoactivated state (Table 1). It is the first time when genotoxicity of photoactivated HYP was studied by the Ames assay.

The molecular background for chromosome aberrations is represented by DSBs, the main sources of which are ionizing irradiation, certain antibiotics and endonucleases. Additional mechanisms leading to DSBs accumulation are DNA replication and DNA excision repair due to accumulated SSBs, mitotic recombination and oxidative damage (Obe et al. 2002). To the best of our knowledge, this was the first time the genotoxic potential of photoactivated HYP was examined on the level of chromosome aberrations (Table 2), which arise as a result of inappropriate repair of DSBs and above mentioned additional mechanisms (Obe et al. 2002). After treating human cell line HepG2 with photoactivated and non-photoactivated HYP, no clastogenic effects were detected respectively. Higher concentrations (0.1–1.0 μg/ml) of photoactivated HYP led to absolute absence of accessible mitosis, because HYP potential clastogenicity was overlaped by its toxicity.

Cytotoxic effect of HYP was studied by several researchers. It is already known that cytotoxic effect of HYP is substantially enhanced by light activation (Thomas and Pardini, 1992). Moreover, the cytotoxicity of HYP also depends on cell line used (Vandenbogaerde et al. 1997). Schmitt et al. (2006) found out that *H. perforatum* extracts containing the photo-activated HYP exhibited less phototoxicity than pure photo-activated HYP. These observations indicate that HYP in *H. perforatum* extract may exert less toxicity than pure HYP due to the presence of additional constituents in the extract.

Thus, research aimed at potential genotoxicity evaluation of photoactivated HYP is of particular interest, mainly due to its exploitation in PDT. In the present study, only the results detected with comet assay point to the potential photogenotoxicity of HYP. However, in the case of comet assay, the positive results could be attributed to the fact that the transient promutagenic DNA changes, e.g. SSBs, might be quickly repaired (Azqueta et al. 2014). Hence, these photogenotoxic findings only have an informative value, in contrast to gene and chromosome mutations.

Although HYP-mediated PDT is already being used in practice, the potential genotoxicity of the compound has not yet been (fully) disproved. Therefore, we believe our results provide a unique advance in knowledge and will be particularly significant for further application of HYP in PDT.

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References

Agostinis P., Vantieghem A., Merlevede W., de Witte P. A. (2002): Hypericin in cancer treatment: more light on the way. Int. J. Biochem. Cell Biol. **34,** 221–241

Ahn J. C., Biswas R., Mondal A., Lee Y. K., Chung P. S. (2014): Cisplatin enhances the efficacy of 5-aminolevulinic acid mediated photodynamic therapy in human head and neck squamous cell carcinoma. Gen. Physiol. Biophys. **33,** 53–62

Azqueta A., Slyskova J., Langie S. A., O'Neill Gaivão I., Collins A. (2014): Comet assay to measure DNA repair: approach and applications. Front. Genet. **5,** 288

Castano A. P., Demidova T. N., Hamblin M. R. (2004): Mechanisms in photodynamic therapy: Partonephotosensitizers, photochemistry and cellular localization. Photodiagn. Photodyn. Ther. **1,** 279–293

Čavarga I., Bilčík B., Výboh P., Záškvarová M., Chorvát D., Kasák P., Mlkvý P., Mateašík A., Chorvátová A., Miškovský P. (2014): Photodynamic effect of hypericin after topical application in the ex ovo quail chorioallantoic membrane model. Planta Med. **80,** 56–62
http://dx.doi.org/10.1055/s-0033-1360174

Collins A. R. (2004): The comet assay for DNA damage and repair. Mol. Biotechnol. **26,** 249–261

Collins A. R., Oscoz A. A., Brunborg G., Gaivão I., Giovannelli L., Kruszewski M., Smith C. C., Stetina R. (2008): The comet assay: topical issues. Mutagenesis **23,** 143–151
http://dx.doi.org/10.1093/mutage/gem051

Feyzioğlu B., Demircili M. E., Özdemir M., Doğan M., Baykan M., Baysal B. (2013): Antibacterial effect of hypericin. Afr. J. Microbiol. Res. **7,** 979–982

Firouzi F., Khoei S., Mirzaei H. R. (2015): Role of resveratrol on the cytotoxic effects and DNA damages of iododeoxyuridine and megavoltage radiation in spheroid culture of U87MG glioblastoma cell line. Gen. Physiol. Biophys. **34,** 43–50

Fox F. E., Niu Z., Tobia A., Rook A. H. (1998): Photoactivated hypericin is an anti-proliferative agent that induces a high rate of apoptotic death of normal, transformed, and malignant T lymphocytes: implications for the treatment of cutaneous lymphoproliferative and inflammatory disorders. J. Invest. Dermatol. **111,** 327–332

Gafrikova M., Galova E., Sevcovicova A., Imreova P., Mucaji P., Miadokova E. (2014): Extract from Armoracia rusticana and its flavonoid components protect human lymphocytes against oxidative damage induced by hydrogen peroxide. Molecules **19,** 3160–3172
http://dx.doi.org/10.3390/molecules19033160

Galloway S. M., Aardema M. J., Ishidate M., Ivett J. L., Kirkland D. J., Morita T., Mosesso P., Sofuni T. (1994): Report from working group on in vitro tests for chromosomal aberrations. Mutat. Res. **312,** 241–261

Garg A. D., Krysko D. V., Vandenabeele P., Agostinis P. (2012): Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. Cancer Immunol. Immunother. **61,** 215–221
http://dx.doi.org/10.1007/s00262-011-1184-2

Gyenge E. B., Forny P., Lüscher D., Laass A., Walt H., Maake C. (2012): Effects of hypericin and a chlorin based photosensitizer alone or in combination in squamous cell carcinoma cells in the dark. Photodiagnosis Photodyn. Ther. **9,** 321–331

Head C. S., Luu Q., Sercarz J., Saxton R. (2006): Photodynamic therapy and tumor imaging of hypericin-treated squamous cell carcinoma. World J. Surg. Oncol. **4,** 87–97

Horváthová E., Slamenová D., Gábelová A. (1999): Use of single cell gel electrophoresis (comet assay) modifications for analysis of DNA damage. Gen. Physiol. Biophys. **18,** 70–74

Hudson J. B., Lopez-Bazzocchi I., Towers G. H. (1991): Antiviral activities of hypericin. Antiviral Res. **15,** 101–112
http://dx.doi.org/10.1016/0166-3542(91)90028-P

Jendželovská Z., Jendželovský R., Hiľovská L., Kovaľ J., Mikeš J., Fedoročko P. (2014): Single pre-treatment with hypericin, a St. John's wort secondary metabolite, attenuates cisplatin- and mitoxantrone-induced cell death in A2780, A2780cis and HL-60 cells. Toxicol. in Vitro **28,** 1259–1273
http://dx.doi.org/10.1016/j.tiv.2014.06.011

Jendželovský R., Mikeš J., Kovaľ J., Souček K., Procházková J., Kello M., Sačková V., Hofmanová J., Kozubík A., Fedoročko P. (2009): Drug efflux transporters, MRP1 and BCRP, affect the outcome of hypericin-mediated photodynamic therapy in HT-29 adenocarcinoma cells. Photochem. Photobiol. Sci. **8,** 1716–1723

Karioti A., Bilia A. R. (2010): Hypericins as potential leads for new therapeutics. Int. J. Mol. Sci. **11,** 562–594

Kersten B., Zhang J., Brendler-Schwaab S. Y., Kasper P., Müller L. (1999): The application of the micronucleus test in Chinese hamster V79 cells to detect drug-induced photogenotoxicity. Mutat. Res. **445,** 55–71

Khan M. A., Cross F. T., Buschbom R. L., Brooks A. L. (1995): Inhaled radon-induced genotoxicity in Wistar rat, Syrian hamster, and Chinese hamster deep-lung fibroblasts in vivo. Mutat. Res. **334,** 131–137

Koval J., Mikes J., Jendzelovský R., Kello M., Solár P., Fedorocko P. (2010): Degradation of HER2 receptor through hypericin-mediated photodynamic therapy. Photochem. Photobiol. **86,** 200–205
http://dx.doi.org/10.1111/j.1751-1097.2009.00639.x

Maron D. M., Ames B. N. (1983): Revised methods for the Salmonella mutagenicity test. Mutat. Res. **113,** 173–215

Miadokova E., Chalupa I., Vlckova V., Sevcovicova A., Nadova S., Kopaskova M., Hercegova A., Gasperova P., Alfoldiova L., Komjatiova M., Csanyiova Z., Galova E., Cellarova E., Vlcek D. (2010): Genotoxicity and antigenotoxicity evaluation of non-photoactivated hypericin. Phytother. Res. **24,** 90–95

Mikeš J., Kovaľ J., Jendželovský R., Sačková V., Uhrinová I., Kello M., Kuliková L., Fedoročko P. (2009): The role of p53 in the efficiency of photodynamic therapy with hypericin and subsequent long-term survival of colon cancer cells. Photochem. Photobiol. Sci. **8,** 1558–1567

Miskovsky P. (2002): Hypericin – a new antiviral and antitumor photosensitizer: mechanism of action and interaction with biological macromolecules. Curr. Drug Targets **3,** 55–84
http://dx.doi.org/10.2174/1389450023348091

Obe G., Pfeiffer P., Savage J. R., Johannes C., Goedecke W., Jeppesen P., Natarajan A. T., Martínez-López W., Folle G. A., Drets M. E. (2002): Chromosomal aberrations: formation, identification and distribution. Mutat. Res. **504,** 17–36

Schmitt L. A., Liu Y., Murphy P. A., Petrich J. W., Dixon P. M., Birt D. F. (2006): Reduction in hypericin-induced phototoxicity by Hypericum perforatum extracts and pure compounds. J. Photochem. Photobiol. B. **85,** 118–130

Šemeláková M., Mikeš J., Jendželovský R., Fedoročko P. (2012): The pro-apoptotic and anti-invasive effects of hypericin-mediated photodynamic therapy are enhanced by hyperforin or aristoforin in HT-29 colon adenocarcinoma cells. J. Photochem. Photobiol. B. **117,** 115–125

Solár P., Ferenc P., Kovaľ J., Mikeš J., Solárová Z., Hrčková G., Fulton B. L., Fedoročko P. (2011): Photoactivated hypericin induces down-regulation of HER2 gene expression. Radiat. Res. **175,** 51–56

Thomas C., Pardini R. S. (1992): Oxygen dependence of hypericin-induced phototoxicity to EMT6 mouse mammary carcinoma cells. Photochem. Photobiol. **55,** 831–837 http://dx.doi.org/10.1111/j.1751-1097.1992.tb08531.x

Traynor N. J., Beattie P. E., Ibbotson S. H., Moseley H., Ferguson J., Woods J. A. (2005): Photogenotoxicity of hypericin in HaCaT keratinocytes: implications for St. John's Wort supplements and high dose UVA-1 therapy. Toxicol. Lett. **158,** 220–224

Turek B., Barta I., Smerak P., Kovacova E., Sedmikova M., Sestakova H. (1997): Mutagenic activity of substances of plant origin. Czech J. Food Sci. **15,** 271–288

Vandenbogaerde A. L., Cuveele J. F., Proot P., Himpens B. E., Merlevede W. J., de Witte P. A. (1997): Differential cytotoxic effects induced after photosensitization by hypericin. J. Photochem. Photobiol. B. **38,** 136–142

Vandenbogaerde A. L., Kamuhabwa A., Delaey E., Himpens B. E., Merlevede W. J., de Witte P. A. (1998): Photocytotoxic effect of pseudohypericin versus hypericin. J. Photochem. Photobiol. B. **45,** 87–94

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