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# Hypericin and Photodynamic Treatment do not Interfere with Transport of Vitamin C during Respiratory Burst

HILDEGARD LAGGNER*, SONJA SCHMID and HANS GOLDENBERG

*Department of Medical Chemistry, Medical University of Vienna, Währinger Strasse 10, A-1090 Vienna, Austria*

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Hypericin is a photosensitizing pigment found in St. John's wort (Hypericum perforatum) displaying a high toxicity towards certain tumors. The fact that some non-tumor cells, especially monocytes and granulocytes, are resistant to its photocytotoxic effects, posed the question whether this insensitivity is due to their ability to accumulate vitamin C, an antioxidant which alleviates the deleterious work of free radicals.

HL-60 promyelocytic tumor cells can be differentiated to neutrophilic granulocytes by treatment with dimethylsulfoxide and were used as cell model. In the differentiated cells, treatment with phorbol esters (PMA) stimulates vitamin C (ascorbate) transport. The uptake rates were unaltered by hypericin at concentrations below 1 μM and irradiation with visible light at a light dose of 6 J/cm$^2$. Inhibition by higher concentrations of hypericin was most probably due to a combination of photocytotoxic properties of the dye and oxygen radicals generated during respiratory burst. Superoxide production by NADPH oxidase followed by reduction of ferricytochrome c was inhibited by hypericin. The degree of inhibition was dependent on the concentration of hypericin and light intensity: $IC_{50}$-values were 1.7 and 0.7 μM under light doses of 3.6 and 10.8 J/cm$^2$, respectively. Oxidative stress, monitored with 2′,7′-dichlorofluorescein (DCF) was only slightly decreased by ascorbate even at higher concentrations of hypericin. In contrast to its effect on the ferricytochrome c-reduction, irradiation had no significant influence on DCF-fluorescence. However, the viability of the cells was strongly decreased after photosensitization and no significant improvement was obtained by ascorbate.

Results from this work indicate that ascorbate transport *per se* is not altered during photodynamic therapy and vitamin C does not interfere with hypericin-induced photodamage of cellular targets.

*Keywords*: Vitamin C; Hypericin; Respiratory burst; HL-60; Superoxide

## INTRODUCTION

In recent years increased interest in hypericin as a potential clinical anticancer agent has grown. The cellular and molecular effects of this substance are well described.[1] The photodynamic dye is preferentially enriched in tumor cells, where it mainly localizes in membranes (endoplasmic reticulum and Golgi, nuclear and plasma membranes and lyzosomes).[2] When photoactivated, hypericin interferes with cell proliferation and signal transduction, however, it is cytotoxic and induces apoptosis and necrosis.[3,4] In this context, the initation of free radical chain reactions is currently the most likely explanation for its efficacy.[5]

One negative side effect of photodynamic cancer treatment is that other non-tumor cells are prone to photosensitive damage too. On the other hand, it has been described that monocytes and granulocytes are relatively resistant to photosensitive damage.[6] One reason for this low sensitivity could rest in the ability of these cells to store large amounts of the important antioxidant vitamin C.[7,8]

Vitamin C is accumulated in neutrophilic granulocytes and related cells by two distinct mechanisms. The reduced ascorbate ion is transported across the cell membrane by a high affinity, low capacity sodium-dependent vitamin C transporter system (SVCTs).[9,10] For dehydroascorbic acid (DHA), a low affinity, high capacity sodium-independent transport system exists including several members of the facilitative glucose transporter family (GLUTs).[11–14]

*Corresponding author. Tel.:+43-1-4277-60811. Fax:+43-1-4277-60881. E-mail: hildegard.laggner@meduniwien.ac.at

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DHA is immediately reduced back to ascorbate within the cells and stored.[15,16]

On activation of the respiratory burst, NADPH oxidase produces superoxide anions as precursors of various reactive oxygen species (ROS). Superoxide oxidizes ascorbate very efficiently to DHA which is preferentially taken up by the cells. Thus, vitamin C might protect the cells from damage by aggressive oxidants and thereby increase their defense efficiency.[17,18]

Hypericin is known to inhibit the neutrophilic respiratory burst oxidase[19,20] probably via the inhibition of protein kinase C. So far, nothing is known about the interplay of vitamin C and hypericin during photosensitization. The purpose of this work is to study the effects of photodynamic treatment of native and differentiated HL-60 cells on the transport of vitamin C.

In this study we provide evidence that hypericin does not influence vitamin C status in HL-60 cells and accumulated ascorbate does not affect photodamage of these cells.



## MATERIALS AND METHODS

Hypericin was obtained from Carl Roth (Karlsruhe, Germany), 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA), at phorbol 12myristate 13-acetate (PMA) and diphenylene iodonium chloride (DPI) were from Calbiochem (San Diego, CA, USA). Hypericin was solubilized in DMSO to 5 mM and stored at 4°C. Stock solutions of $H_2$DCF-DA (60 mM in dimethylformamide), PMA (500 μg/ml DMSO) and DPI (5 mM in DMSO) were stored at −20°C. All stock solutions were freshly diluted in buffer immediately prior to use.

All other chemicals were from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland) if not stated otherwise.

### Cell Culture

HL-60 promyelocytic tumor cells were cultured in RPMI 1640-media (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (Gibco, NY, USA), 2 mM glutamine, 50 U/ml streptomycin and 0.1 mg/ml penicillin (PAA-Laboratories, Linz, Austria) and were kept in a humidified incubator in a 5% $CO_2$ atmosphere at 37°C. Cell viability was determined by trypan blue exclusion or neutral red retention (see below). For experiments with native HL-60 cells and to start differentiation, only cells with viability higher than 95% were used. Neutrophilic differentiation of the cells was performed by incubation with 1.3% (v/v) dimethylsulfoxide (DMSO) for 6 days.[21] The differentiated state was verified by measuring the superoxide-dependent reduction of ferricytochrome c after stimulation with 160 nM PMA.[22]

### Cell Photosensitization

Cells were preincubated with various concentrations of hypericin for 10 min at 37°C and subsequently irradiated with visible light (50 W halogen lamp, OSRAM, München, Germany). At the surface of the sample tubes, the fluence rate was between 6 and 10 mW/cm$^2$ as measured with an IL 1800 radiometer (International Light, Newburyport, MA, USA). The light dose was calculated from the exposure time and was between 3.6 and 10.8 J/cm$^2$.

### Uptake Studies of Vitamin C

Cells were harvested by centrifugation at 200*g* at 37°C for 10 min, washed twice with prewarmed Tris/saline/glucose buffer (TBSG; 140 mM NaCl, 20 mM Tris, 5 mM glucose, pH 7.4) and resuspended at a final concentration of $2.5 \times 10^6$ cells/500 μl. All additions used for the specific experiments were included in this volume.

After preincubation with hypericin for 10 min, irradiation and stimulation of the respiratory burst with 160 nM PMA for 5 min, the experiment was started by the addition of ascorbate. When DHA was used as substrate, 2 U of ascorbate oxidase were added and complete oxidation was verified by HPLC. To test the effect of superoxide on ascorbate uptake, the radical generating system xanthine/xanthine oxidase (X/XO) was used. For this, cells were preincubated with 200 μM xanthine for 5 min, then ascorbate was added and the assay was started by the addition of 25 mU xanthine oxidase.

The uptake experiments were terminated at various time points by addition of 1 ml ice-cold buffer and centrifugation through a layer of oil [dibutylphthalate/dioctylphthalate (4:1, v/v)] at 1000*g* for 60 s. For determination of external vitamin C content, an aliquot of supernatant was mixed with an equal volume of 10% (w/v) meta-phosphoric acid (MPA). Residual supernatant and oil were aspirated and 500 μl 5% (w/v) MPA were added to the cell pellet. The samples were either analyzed immediately by HPLC or frozen at −80°C.

### Determination of Vitamin C by HPLC

Ascorbate and DHA concentrations were measured by HPLC and electrochemical detection according to the method of Lykkesfeldt *et al.*[23] Briefly, the MPA-treated samples were thawed, cells were homogenized with an ultrasound pulse (Branson sonifier, Danbury, CT, USA) for 3 s on ice, diluted with the same volume of 125 mM Tris and injected into

the HPLC system (JASCO, Tokyo, Japan) with the help of a thermostated autosampler at 4°C. Separation of ascorbic acid was carried out on a Spherisorb ODS-2 column (125 × 4.6 mm, 3 μ) with an ODS-Hypersil precolumn (Dr. A. Maisch, Ammerbuch, Germany). The mobile phase consisted of 100 mM $Na_2HPO_4$, 2.5 mM $Na_2EDTA$, 2 mM n-dodecyl trimethylammonium chloride, adjusted to pH 3 with ortho-phosphoric acid. A flow rate of 1 ml/min was used. The electrochemical detector was from Bioanalytical Systems (West Lafayette, IN, USA) and the glass carbon electrode was set at 600 mV against an Ag/AgCl-reference. Data was recorded and processed using BORWIN Chromatography software, Version 1.5.

To estimate the concentration of DHA, 500 μl of MPA-treated samples were reduced to ascorbic acid by addition of 250 μl of 10 mM dithiothreitol (DTT) in 0.5 M Tris for 10 min. After reacidification with 250 μl of 0.2 M sulphuric acid, the samples were analyzed by HPLC and DHA was taken as the difference between the concentrations obtained in the presence and in the absence of DTT.[24]

### Measurement of Superoxide by Ferricytochrome C

The $5 \times 10^6$ differentiated HL-60 cells per ml TBSG-buffer were preincubated with increasing concentrations of hypericin for 10 min at 37°C, then irradiated with a light dose of 3.6 or 10.8 J/cm$^2$, stimulated with 160 nM PMA and reduction of ferricytochrome c (50 μM) was followed at 550 nm over a time period of 10 min in a spectrophotometer equipped with a water jacketed cell holder (Lambda 2, Perkin Elmer, Fremont, CA, USA) set at 37°C.

### Measurement of Oxidative Stress by $H_2$DCF-DA

Cells were plated in black flat-bottom 96 well plates (Greiner bio-one, Kremsmünster, Austria) at a density of $5 \times 10^5$ cells per well in 100 μl TBSG that contained various concentrations of hypericin. After a preincubation of 10 min, generation of superoxide with either PMA or X/XO and photosensitization, 100 μl of 2′7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) was added (50 μM).[25] Upon reaction with oxidizing species, the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) was formed and light emission was followed in an automated plate fluorescence reader (Wallace, Turku, Finland) at 37°C using an excitation wavelength of 485 nm. Emission was recorded at 535 nm every 5 min over a time period of 90 min.

### Cell Viability

Neutral red was dissolved in water (0.4%), diluted (1:80) in unsupplemented RPMI 1640-media and added to the cells immediately after photosensitization. Control and treated cells were incubated with the dye for 3 h in 96 well plates in an incubator with 5% $CO_2$ atmosphere and then washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$.12 $H_2O$, pH 7.4), lyzed in 50% (v/v) ethanol/1% (v/v) acetic acid for 30 min and analyzed in a plate reader at 540 nm. Corrections were made for nonspecific absorption of neutral red in buffer, in the absence and presence of hypericin.

### Data Analysis

All experiments were performed in triplicates if not stated otherwise and repeated at least three times to ensure reproducibility. Results are shown as mean values ± S.E.M. Statistical analysis were performed by the Student *t*-test and differences were considered to be significant if $p < 0.05$.

## RESULTS

Promyelocytic HL-60 tumor cells can be differentiated into neutrophilic granulocytes by treatment with dimethylsulfoxide and show many morphological, functional and biochemical similarities with normal peripheral blood granulocytes.[26]

### Inhibition of Superoxide Production by Hypericin

Differentiated HL-60 cells responded to activation by phorbol 12-myristate 13-acetate (PMA) via the generation of superoxide. Production rates between 1 and 2 nmol/min/$10^6$ cells were measured by superoxide dismutase (SOD)-inhibitable ferricytochrome c-reduction. After preincubation with hypericin with subsequent irradiation with visible light, a concentration- and light-dependent inhibition of superoxide generation was observed (Fig. 1). The inhibitory concentration ($IC_{50}$) was 1.7 μM at an irradiation dose of 3.6 J/cm$^2$ and 0.7 μM at 10.8 J/cm$^2$. No effect on PMA-induced superoxide generation was observed under light-protected conditions or upon illumination without hypericin or without preincubation with the dye.

When the superoxide-generating system X/XO (200 μM and 25 mU) was used, no inhibition by hypericin was monitored (data not shown).

From these data we conclude that only following incubation with hypericin and photodynamic treatment, superoxide production from activated NADPH oxidase is reduced.

### Inhibition of Ascorbate Uptake by Hypericin

The uptake of ascorbate was strongly increased in differentiated HL-60 cells after activation with PMA

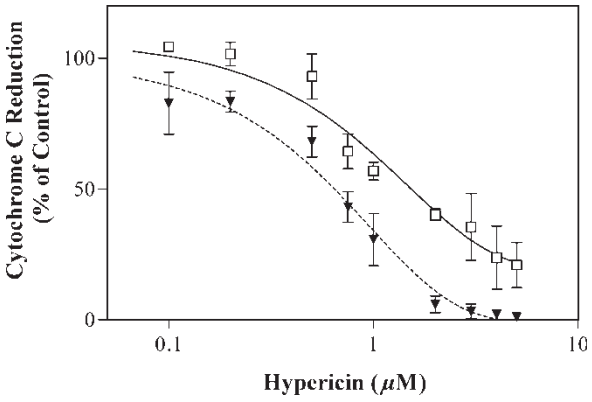



FIGURE 1 Effect of hypericin on superoxide production in differentiated HL-60 cells. $5 \times 10^6$ cells/ml were preincubated at 37°C with increasing concentrations of hypericin for 10 min. The reaction mixture was then exposed to light from a 50 W halogen lamp with a fluence rate of 10 mW/cm$^2$ for 10 or 30 min to give a radiation dose of 3.6 (squares) and 10.8 J/cm$^2$ (triangles) as measured with an IL 1800 radiometer (International Light, MA, USA). Results are means ± S.E.M. for five experiments.

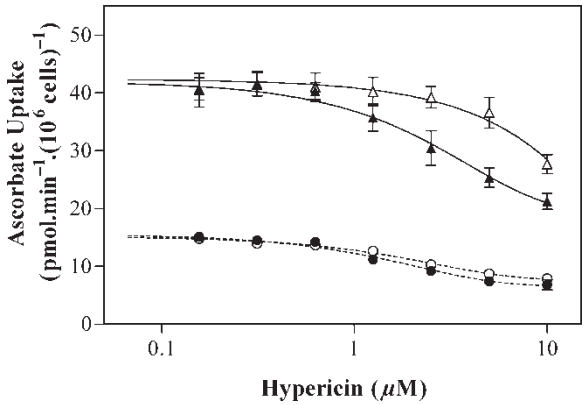



FIGURE 2 Inhibitory effect of hypericin on the uptake rates of ascorbate. Cells were preincubated with increasing concentrations of hypericin for 10 min and irradiated with a light dose of 6 J/cm$^2$ (closed symbols) or kept under dark conditions (open symbols). Differentiated HL-60 cells were stimulated with 160 nM PMA (triangles) and native HL-60 cells were incubated with 200 μM xanthine and 25 mU xanthine oxidase (circles). Results are means ± S.E.M. for three experiments.

and the uptake rates were close to those found when the oxidized vitamin C (DHA) was added as substrate (Table I). These data indicate that during respiratory burst, ascorbate was most probably oxidized by superoxide anions to DHA which is then transported into the cells. Ascorbate uptake in native as well as in differentiated HL-60 cells could also be stimulated with X/XO. However, the addition of 200 μM xanthine together with 25 mU xanthine oxidase enhanced the uptake rate for native HL-60 cells three-fold at the most. Using a starting concentration of 100 μM, the uptake rate increased from 5.1 ± 1.1 to 15 ± 0.9 pmol/min/$10^6$ cells. No DHA could be detected intracellularly confirming the fast reduction to ascorbate.

The stimulated ascorbate uptake was inhibited by hypericin depending on the concentration and exposure to light. Inhibition was also seen in the dark but to a lower extent as after irradiation with a light dose of 6 J/cm$^2$ (Fig. 2).

When we compared the effect of 1 μM hypericin on ascorbate uptake, we found a decrease of around 10% only in stimulated vitamin C transport and after irradiation (Fig. 3A). Xanthine/xanthine oxidase enhanced ascorbate uptake around three-fold as already seen with native cells ($p < 0.001$).

TABLE I Uptake rates of 100 μM ascorbate and DHA in differentiated HL-60 cells with and without activation of the respiratory burst by 160 nM PMA

| PMA | Ascorbate | DHA | Uptake rates (pmol/min/$10^6$ cells) |
|---|---|---|---|
| − | + | − | 3.9 ± 1.4 |
| + | + | − | 41.7 ± 12 |
| − | − | + | 45.3 ± 10 |

Vitamin C content was measured by HPLC and electrochemical detection. Results are means ± S.E.M. for five experiments.

With a starting concentration of 100 μM ascorbate, the uptake rate increased from 4 ± 0.4 to 13.7 ± 1.5 pmol/min/$10^6$ cells and was lowered to 12.3 ± 1.6 pmol/min/$10^6$ cells by 1 μM hypericin. A 10-fold increase to 40.4 ± 6.4 pmol/min/$10^6$ cells due to PMA-stimulation could be observed ($p < 0.001$) with a decline to 35.7 ± 7.4 pmol/min/$10^6$ cells with 1 μM hypericin. No differences were observed after preincubation with the dye at basal uptake of ascorbate or when stimulated cells were not exposed to light.

Next, we measured the residual extracellular concentrations of ascorbate and DHA after an uptake period of 15 min. Nearly full initial concentration could be recovered extracellularly when basal ascorbate uptake was monitored (Fig. 3B). Even though ascorbate is known to be light sensitive, it was not destroyed during irradiation, as shown by the complete retrieval. When DHA was measured as the difference between ascorbate and total vitamin C after reduction with dithiothreitol, less then 1% of the initial concentration was detected as DHA which is not significant (Fig. 3C). With X/XO, only around 70% of ascorbate was still in the reduced form. The decrease of external ascorbate was not a result from the increase of intracellular ascorbate because only around 0.5 nmol from the initial mass of 50 nmol were accumulated within the total $2.5 \times 10^6$ cells after 15 min. Concentrations of DHA around 10% were measured extracellularly. Therefore, we concluded that ascorbate was oxidized by superoxide from X/XO to DHA and either transported into the cells (as seen in Fig. 3A) or further oxidized.

However, external ascorbate was lowest with PMA (Fig. 3B) and only around half of the initial concentration of ascorbate was detectable in the reduced form and again around 10% were measured

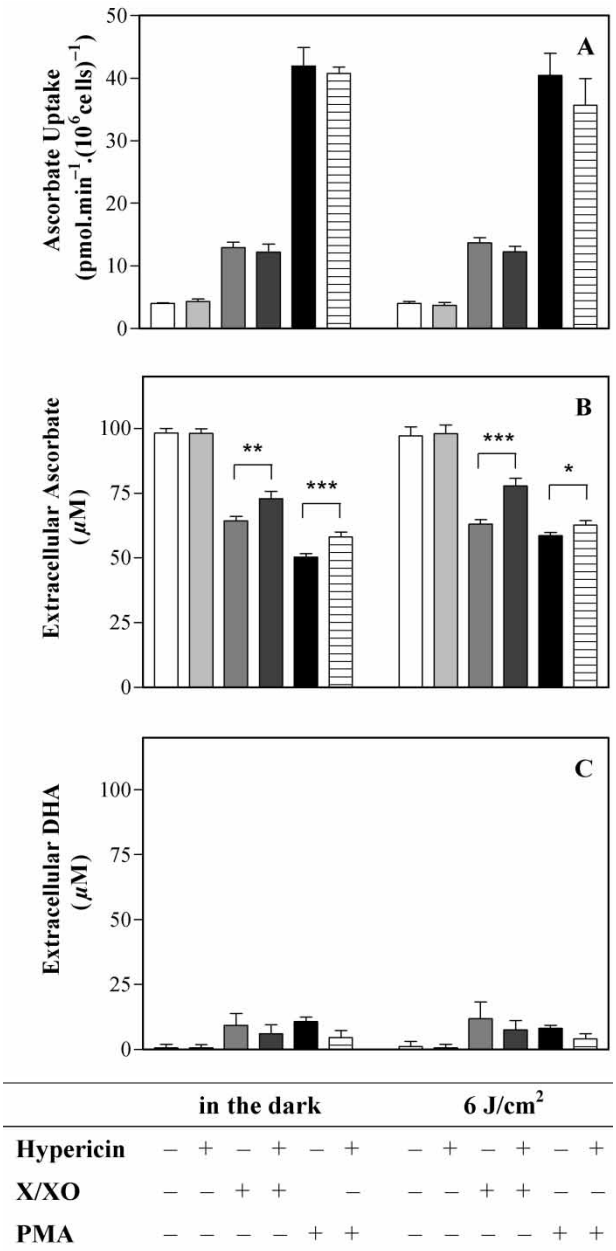



FIGURE 3 Effect of 1 μM hypericin on uptake rates of 100 μM ascorbate in differentiated HL-60 cells (A), extracellular concentrations of ascorbate (B) and DHA (C) after 15 min incubation period. Differentiated HL-60 cells were preincubated with 1 μM hypericin and irradiated with a light dose of 6 J/cm$^2$ or kept under light-protected conditions. Activation of ascorbate uptake was performed by addition of 160 nM PMA or 200 μM xanthine with 25 mU xanthine oxidase (X/XO). DHA in panel C was calculated from the difference of vitamin C content measured with and without DTT as described under "Materials and Methods" section. Results are means ± S.E.M. for three experiments. (A) $p < 0.001$ for all stimulated groups compared with corresponding unstimulated controls. (B) ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

as DHA (Fig. 3C). In the presence of PMA, 3% of initial ascorbate was found within the cells. There was still a significant loss of vitamin C that can occur via further irreversible oxidation.

In the presence of hypericin, higher extracellular concentrations of ascorbate ($p < 0.05$) and slightly lower concentrations of DHA were found no matter if superoxide was produced with X/XO or after stimulation with PMA. Interestingly, no differences in the nature and content of extracellular ascorbate and DHA were observed between irradiation and dark conditions.

### Effect of Hypericin on DCF-fluorescence and Viability of PMA-stimulated HL-60 Cells

As additional marker for oxidative stress in native and activated cells we used 2′,7′dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) (Fig. 4A,B). Fluorescence of 2′,7′dichlorofluorescein (DCF) was strongly increased after activation of the respiratory burst with PMA but less inhibited by hypericin than ferricytochome c reduction. Inhibition by hypericin was comparable to the decrease of stimulated

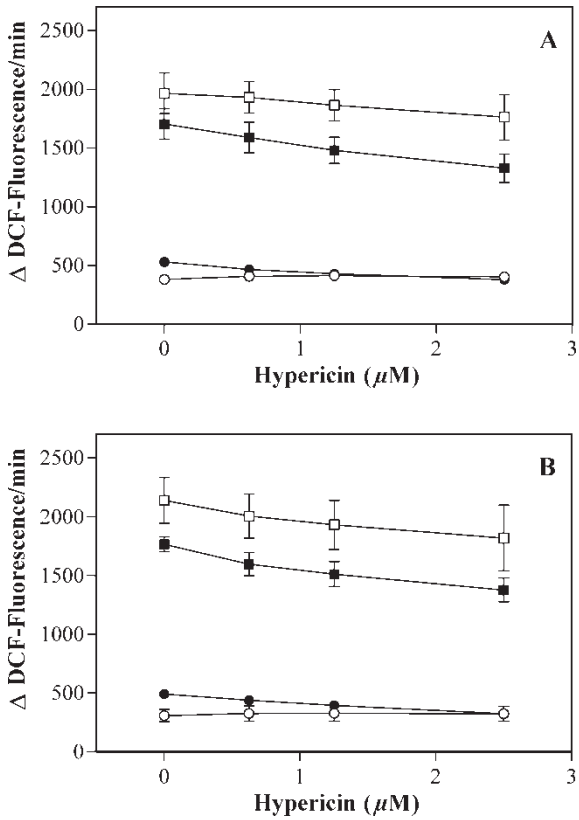



FIGURE 4 Concentration-dependence of hypericin on oxidant level measured by 2′,7′-dichlorofluoresein (DCF). Differentiated HL-60 cells ($5 \times 10^5$ per well) were loaded with 0.625, 1.25 or 2.5 μM hypericin and kept under dark conditions (A) or irradiated with 6 J/cm$^2$ (B). Cells were then stimulated with 160 nM PMA (squares) or kept unstimulated (circles) and incubated without (open symbols) or with 100 μM ascorbate (closed symbols) for another 5 min. The fluorescence intensity of DCF (50 μM) was followed every 5 min in an automated plate reader over a period of 90 min. Excitation/emission wavelengths were 485/535 nm. Samples were measured in five replicates and each experiment was repeated three times.

ascorbate uptake. Ascorbate diminished DCF-fluorescence, but it was lesser than that when native HL-60 cells together with X/XO were monitored (see Fig. 6A). No significant difference could be detected between irradiated and non-irradiated cells. Corrections for DCF-fluorescence from photoactivated hypericin were made whereby 2.5 μM hypericin showed between 5 and 10 relative fluorescence units per min.

Because the photocytotoxic property of hypericin has been known for years, we determined the toxic effect of hypericin with neutral red in parallel with DCF-fluorescence. No toxicity of hypericin in differentiated cells was observed when the concentration was up to 2.5 μM and cells were kept in the dark, no matter if ascorbate was added or not (Fig. 5A). Viability decreased after stimulation with PMA also in the dark and was diminished by hypericin in a concentration-dependent manner. When cells were illuminated, a dramatic decrease in neutral red retention was observed due to hypericin even in unstimulated cells (Fig. 5B). Only a slight protection of viability could be achieved with ascorbate.

### Effect of Hypericin on DCF-fluorescence and Viability of Native HL-60 Cells

To ascertain whether the monitored effects were due to superoxide, we used the radical generating system X/XO together with native HL-60 cells. Under these conditions, nearly a 10-fold increase in DCF-fluorescence was observed. Again, no difference was seen if cells were irradiated (Fig. 6A) or not (data not shown). However, ascorbate strongly diminished DCF-fluorescence confirming the reactivity between ascorbate and superoxide.

Without irradiation, little reduction in viability was observed with increasing concentrations of hypericin (data not shown), whereas an exposure of the cells to a light dose of 6 J/cm$^2$ strongly decreased the viability, which was down to 50% at

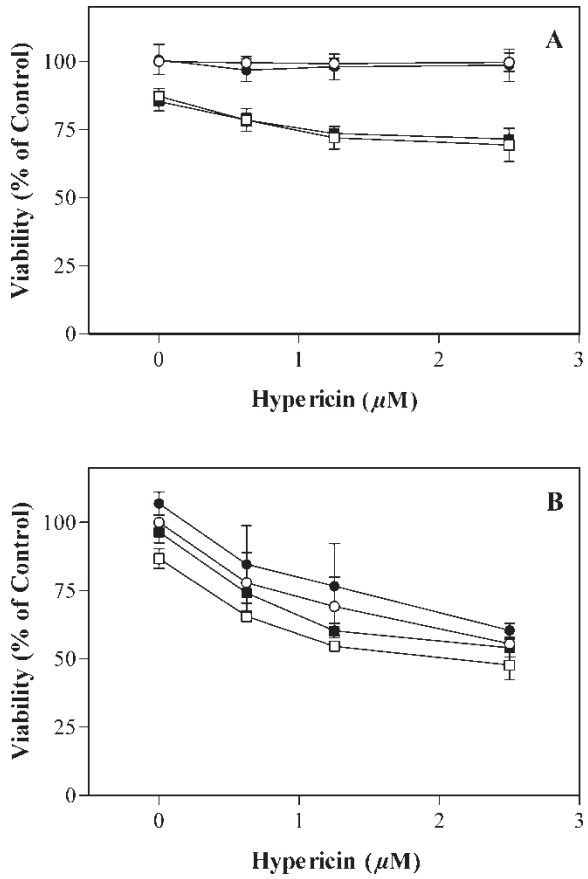



FIGURE 5 Photocytotoxicity of hypericin. Cells were plated in 96 well plates at $5 \times 10^5$ cells per well in 100 μl of buffer that contained the photodynamic reagent without (circles) or with PMA (squares) in the presence (closed symbols) or absence (open symbols) of 100 μM ascorbate before irradiation with 6 J/cm$^2$ (B). Panel A shows the control experiments without irradiation. Neutral red was added immediately after photosensitization incubated with the cells for 3 h and analyzed in a plate reader at 540 nm. Results are means ± S.E.M. for three experiments.

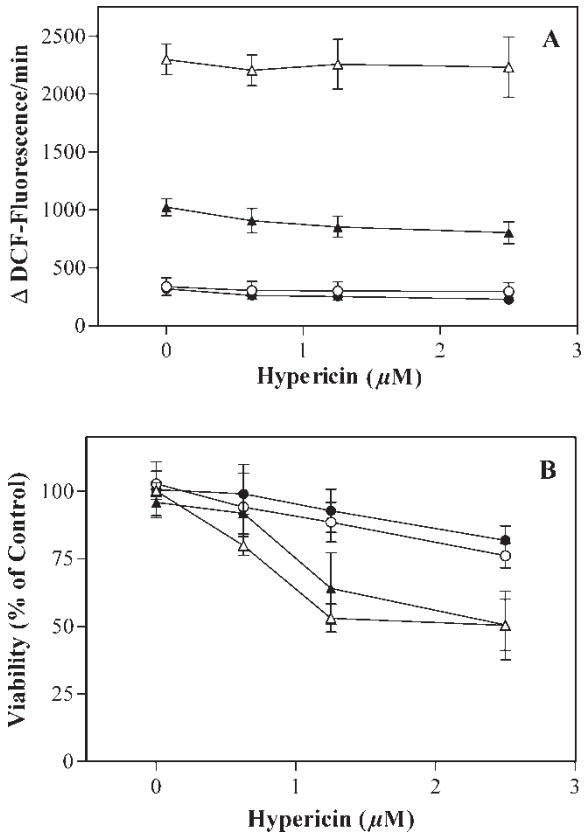
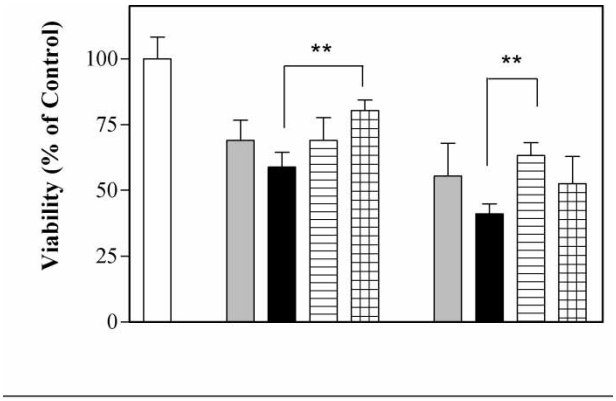



FIGURE 6 DCF-fluorescence and viability of native HL-60 cells after treatment with hypericin, ascorbate and xanthine/xanthine oxidase. Cells were preincubated with various concentrations of hypericin and irradiated with 6 J/cm$^2$. A measured quantity of 100 μM ascorbate (closed symbols), 200 μM xanthine and 25 mU of xanthine oxidase (triangles) were added and DCF-fluorescense was monitored (A). Open symbols were incubations without ascorbate and circles were native cells. Viability was measured as neutral red retention (B). Results are means ± S.E.M. for three experiments.




| | Control | PMA | | | | X/XO | | | |
|---|---|---|---|---|---|---|---|---|---|
| Hypericin | − | − | + | + | + | − | + | + | + |
| SOD/Cat | − | − | − | + | − | − | − | + | − |
| DPI | − | − | − | − | + | − | − | − | + |

FIGURE 7 Effect of xanthine/xanthine oxidase (X/XO), PMA and hypericin on the viability of HL-60 cells measured with the neutral red assay. Cell were preincubated with 10 U SOD and 1000 U catalase/well (SOD/Cat) or 5 μM DPI, treated with 1 μM hypericin, irradiated with 6 J/cm$^2$ and stimulated with PMA or X/XO. Results are means ± S.E.M. for three experiments. $p < 0.05$ for all groups compared with the control, $^{**}p < 0.01$.

1.25 μM hypericin (Fig. 6 B). Ascorbate again showed no significant effect on the loss of viability.

### Increase in Viability due to Inhibition of Superoxide-production

When differentiated cells were stimulated with PMA and exposed to photosensitization, only around 70% of neutral red retention was measured compared to unstimulated cells (Fig. 7). When these differentiated cells were treated with X/XO, the viability sank to around 55%. Preincubation with 1 μM hypericin decreased the viability even further. This photocytotoxicity of hypericin could hardly be abolished by SOD together with catalase after PMA treatment but was more pronounced when X/XO was added to the cells ($p < 0.01$). Diphenylene iodonium chloride as a blocker of NADPH oxidase showed the strongest protective efficacy in PMA-treated cells ($p < 0.01$). No difference was observed when ascorbate was added (data not shown).

These data suggest that a substantial decrease in viability of HL-60 cells under photodynamic treatment with hypericin and respiratory burst was due to the toxicity of superoxide and/or other reactive oxygen radicals. Vitamin C interacted with reactive species but showed no protective effect regarding the phototoxicity of hypericin.

## DISCUSSION

Our experiments indicate that vitamin C does not protect differentiated HL-60 cells from damage due to photoactivated hypericin. These cells can be loaded with ascorbate in the millimolar range upon stimulation with phorbol esters. In our previous work we found that this stimulated uptake is competitively abolished by inhibitors of the DHA transport and is reversed by SOD and catalase. Furthermore, it is sensitive to inhibitors of the respiratory burst as well as the PKC inhibitor staurosporin indicating that ascorbate is oxidized during respiratory burst to DHA which is preferentially taken up into the cells where it is immediately reduced back to ascorbate.[18]

During photodynamic therapy, a specific accumulation of a photosensitizing compound in the tumor cells and subsequent activation with visible light is used. In the presence of oxygen, ROS are generated which can then kill the target tumor cells.

In this work we could show that superoxide production from differentiated HL-60 cells stimulated with PMA was inhibited by hypericin in a light- and concentration-dependent manner when measured by SOD-inhibitable ferricytochrome c reduction. A half-maximal inhibitory concentration around 1 μM was determined after irradiation with visible light whereas no alteration was observed under dark conditions. As hypericin does not interact with superoxide generated from X/XO, we conclude that photoactivated hypericin interacts with the activation of the NADPH oxidase. Conversely, when the oxidant level was followed by reaction with the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF), little inhibitory effect of hypericin can be seen. DCF is widely used to measure oxidative stress in cells, even if which oxidative species are responsible for the oxidation of $H_2$DCF-DA is not clear. As enhanced fluorescence was inhibitable by SOD, at least part of the fluorescence originate from superoxide and its reaction products. Ascorbate inhibited DCF-fluorescence when initiated with X/XO but only modestly after PMA-stimulation. Photodynamic dyes like hypericin are known to be photocytotoxic, but it is not known if decrease in DCF-fluorescence can be masked by intermediates from apoptotis and/or necrosis. Taken together, inhibition of enhanced DCF-fluorescence after PMA-stimulation was less pronounced when oxidative stress was measured by reduction of ferricytochrome c.

Interestingly, the dose-response of hypericin on DCF-fluorescence was very similar to the inhibition on stimulated ascorbate uptake. When we investigated the effect of the photosensitizing dye on the vitamin C accumulation during respiratory burst, we found that vitamin C uptake is not markedly inhibited by concentrations below 1 μM hypericin and only around 10% with 1 μM hypericin.

We conclude that already a minor amount of superoxide is efficient for the enhanced uptake rates

during oxidative burst and that ascorbate uptake is decreased by higher concentration (above 1 μM) mainly due to the celltoxic properties of hypericin, which are further multiplied by the generation of ROS.

To gain further information on the potential cytotoxicity of hypericin, we monitored the retention of neutral red.[27] Uptake of neutral red was unaltered by hypericin under light-protected conditions but declined in a dose-dependent manner in native cells after irradiation. Our data were comparable to those published by Lavie and others[28] who reported cell death of HL-60 with a $LD_{50}$ of 3 and 0.7 μM after radiation dose of 4.8 and 14.4 $J/cm^2$, respectively. Nevertheless, an even more pronounced decrease in viability was monitored in differentiated and stimulated cells. Not all measured activities could be attributed to photoactivated hypericin but was amplified by cell damage due to the respiratory burst. No protective effect of ascorbate on hypericin-induced cytotoxicity was found.

After ascorbate uptake in activated HL-60 cells, there was always a higher residual ascorbate concentration measured when hypericin was added. It is not clear if this is due to some stabilising properties of the dye or the decrease in superoxide production and subsequent ascorbate oxidation.

The fact that primary leukocytes (monocytes and granulocytes) are relatively insensitive to photodynamic therapy led to the hypothesis that the accumulation of the antioxidative vitamin C could be responsible for that inefficacy. In this work, we used differentiated and stimulated HL60 cells as a model for neutrophilic granulocytes during respiratory burst and found that there is certain interplay between ascorbate and hypericin but there is no firm evidence to support this theory.



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