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# Hypericin-Induced Phototoxicity of Human Leukemic Cell Line HL-60 is Potentiated by Omeprazole, an Inhibitor of $H^+K^+$-ATPase and 5′-(N,N-dimethyl)-amiloride, an Inhibitor of $Na^+/H^+$ Exchanger

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## Summary

Hypericin, an antiretroviral and antineoplastic agent, seems to have multiple modes of light-induced biological activity connected with the production of single oxygen and/or excited-state proton transfer and a consequent pH drop of pH formation in the hypericin environment. In the present study omeprazole, an inhibitor of $H^+K^+$-ATPase, and amiloride, an inhibitor of the $Na^+/H^+$ exchanger, have been used for testing the hypothetical pH decreasing effect of hypericin in its antineoplastic action. The results of our experiments have shown that in the HL-60 cell line the effect of hypericin ($10^{-6}$ mol.$l^{-1}$) was significantly potentiated by omeprazole and 5′-(N,N-dimethyl)-amiloride. The effect of omeprazole seemed to be less specific than that of 5′-(N,N-dimethyl)-amiloride. Our results support the hypothesis that the excited-state proton transfer and the consequent acidification of the hypericin environment could play a role in the biological activity of hypericin. Moreover, both omeprazole and 5′-(N,N-dimethyl)-amiloride are effective potentiating agents of hypericin cytotoxic effect in the HL-60 cell line.

**Key words**
Hypericin • Omeprazole • Amiloride • HL-60 • Cytotoxic activity

## Introduction

Hypericin (Fig. 1), a polycyclic quinone, is a naturally occurring pigment found in plants (St. John's Wort – *Hypericum perforatum*). Recent interest in hypericin was spawned by the discovery that it possesses extremely high toxicity towards certain viruses, notably the class of enveloped viruses that includes the human immunodeficiency virus (HIV) (Meruelo *et al.* 1988, Lavie *et al.* 1989, 1990, Kraus *et al.* 1990), as well as towards tumors (Andreoni *et al.* 1994, Zang *et al.* 1996) and that this toxicity is absolutely light-dependent. Other authors have shown that irradiation of hypericin with visible light leads to the production of singlet oxygen (Jardon *et al.* 1987, Racinet *et al.* 1988) or free radicals (Durdan and Song 1986, Yang *et al.* 1986). Consequently, an oxygen-dependent mechanism of hypericin activity has been proposed by Thomas *et al.*



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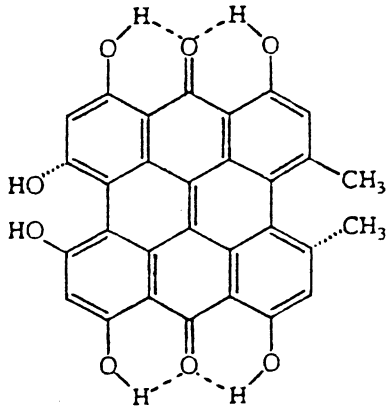
(1992). However, it has recently been reported that although in some cases it may play a role, oxygen is not always required for the antiviral activity of hypericin (Fehr *et al.* 1994). In addition,. an alternative origin of the photoinduced antiviral activity has been suggested by Petrich and coworkers namely that it is able to produce a photogenerated pH drop (Fehr *et al.* 1995a). From the point of view of the mechanism of hypericin's action in cells, pH decrease caused by illumination of 3T3 mouse fibroblasts containing hypericin is very interesting. In their study, using an SNARF-1 pH fluorescence probe, Miskovsky and coworkers have found a local diminution of pH, which depends on the intensity of light (Sureau *et al.* 1996). This result, which has been suggested to be related to the biological activity of hypericin (Fehr *et al.* 1995a,b,) supports the observation of Petrich's group about the local pH decrease after illumination (Fehr *et al.* 1995a).

**Fig. 1.** *Structure of hypericin.*




Intracellular pH is the crucial factor governing the activity of intracellular enzymes. Almost all cellular processes can be affected by changes in intracellular pH. Intracellular pH changes seem to be important in controlling the cell cycle and the proliferative capacity of cells, in the action of growth factors, hormones, neurotransmitters and drugs, the efficacy of contractile elements, the conductivity of ionic channels and subsequent cell survival (Madshus 1988).

Omeprazole is a prodrug which is converted by acid to its active form, a sulphenamide (Sachs *et al.* 1988). As omeprazole is a weak base, it becomes concentrated in cellular spaces where the pH is more acidic. The principal target of omeprazole is a proton pump, $H^+K^+$-ATPase of gastric parietal cells, but it also inhibits the microsomal isoform of the $H^+K^+$-ATPase in mammalian renal medulla (Sabolic *et al.* 1994). Moreover, it has been reported that omeprazole also inhibits the vacuolar type of $H^+$-ATPase, i.e. the $H^+$ pump, in intracellular renal cortical and medullary endocytic vesicles (Sachs *et al.* 1988) and osteoclast-derived vesicles, but with a lower potency in comparison with the gastric proton pump (Mattsson *et al.* 1991).

Another substance influencing intracellular pH by a more specific mechanism is amiloride. It is an inhibitor of membrane-associated $Na^+/H^+$ exchanger. This exchanger responds to cellular acidification by extruding the $H^+$ ion in exchange for the influx of the $Na^+$ ion (Madshus 1988). In our experiments we used 5′-(N,N-dimethyl)-amiloride to inhibit this exchanger and to test its activity in combination with hypericin.

The main goal of the present paper was to determine the possible effect of proton pump inhibiting substance, omeprazole, and membrane ion exchanger inhibitor, amiloride, on light-induced hypericin cytotoxicity to HL-60 cells. Consequently, we investigated the hypothesis concerning the biological activity of hypericin linked to its ability to produce a photogenerated pH drop.

## Methods

*Cell culture*

The HL-60 promyelocytic cell line was kindly provided by Dr. M. Hajdúch (Olomouc, Czech Republic). This cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal calf serum (FCS), 25 mg.100 $ml^{-1}$ glutamine and penicillin/streptomycin (100 I.U.$ml^{-1}$ and 100 μg.$ml^{-1}$, respectively).

*Drugs*

Hypericin (Carl-Roth, Germany) was dissolved in dimethyl sulfoxid hybri max (DMSO) and diluted to final concentrations with DMEM (Sigma, Germany) used for cell culture techniques. The definitive concentration of DMSO (Sigma, Germany) in culture wells never exceeded 0.25 %, which did not influence the cell viability (data not shown). Omeprazole was used in the form of injection (Losec, Astra, Sweden), freshly dissolved and adjusted to the desired concentrations with DMEM. The substance of 5′-(N,N-dimethyl)-amiloride (DMA, Sigma) was dissolved and diluted to the desired concentration with DMEM.

*Illumination*

A power-controlled low intensity halogen lamp was used as a light source in the experiment. The cell cultures were illuminated with a light dose of 0.4 J/cm$^2$. Complete light spectrum was used for illumination.

*Experimental procedure*

The thiazolyl blue (MTT) method was used in all experiments for the assessment of the cytotoxic effect of tested agents (Mosmann 1983, Mihál *et al.* 1995). Briefly, cell suspensions containing 4x10$^4$ viable cells *per* vial were cultivated in 96-well tissue culture plates with or without the tested substances for three days. The cells were cultivated at 37 °C in a humidified 5 % $CO_2$ air atmosphere. After 72 hours of cultivation, MTT (Sigma, Germany) was added to each sample and the cultivation continued for additional 4 h. During this period the living cells produced blue insoluble formasan from the yellow soluble MTT. The reaction was stopped by addition of 100 μl of 10 % laurylsulfate in each well and the content of the wells was spontaneously dissolved within the following 12 h. The optic density of each well was measured spectrophotometrically at 540 nm on ELISA reader MRX Dynatech (Great Britain). The obtained values (n=10) were calculated and expressed as the percentage of cell survival in comparison with the controls taken as 100 % survival.

Student's t-test was applied to evaluate these growth experiments. P<0.001 was taken as significant.

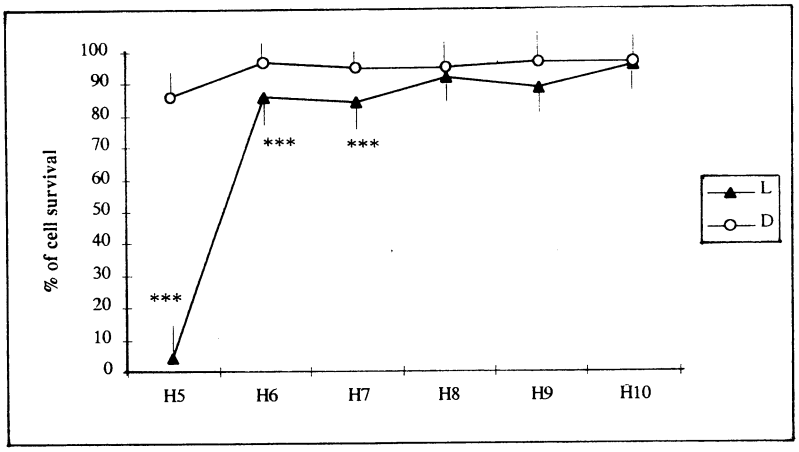
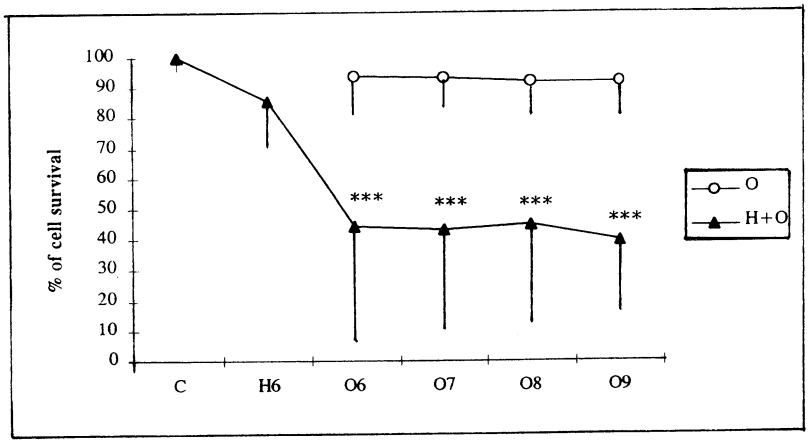



**Fig. 2.** *The effect of hypericin in concentrations from 10$^{-5}$ to 10$^{-10}$ mol.l$^{-1}$ (H5-H10) on promyelocytic HL-60 cell line survival in dependence on light (L) and dark (D) conditions. The effect of hypericin is significantly higher after illumination from 10$^{-5}$ to 10$^{-7}$ mol.l$^{-1}$ concentration. Significant differences from cells cultivated in dark conditions are expressed as ***P<0.001.*

**Fig. 3.** *The effect of omeprazole alone (O) and in combination with hypericin (H+O) on cell survival. C – control cells without any treatment; H6 – cells treated with hypericin alone at 10$^{-6}$ mol.l$^{-1}$;O6-O9 – omeprazole at 10$^{-6}$ to 10$^{-9}$ mol.l$^{-1}$ in combination with hypericin at 10$^{-6}$ mol.l$^{-1}$. Significant differences versus hypericin alone are expressed as ***P<0.001.*




## Results

Since the cytotoxic effect of hypericin requires illumination. the experiments were performed under light conditions. Parallel experiments in the dark revealed significant differences in cell survival between these two procedures (Fig. 2). The highest cytotoxic effect of hypericin was observed in the 10$^{-5}$ mol.l$^{-1}$ concentration, significant effects at 10$^{-6}$ and 10$^{-7}$ mol.l$^{-1}$.

In the initial experiments, omeprazole alone was also tested under both light and dark conditions to determine its effect on cells. The results did not reveal any significant differences between the light and dark conditions (data not shown). Moreover, they demonstrated that omeprazole alone did not exert any cytotoxic effects in the tested concentrations (Fig. 3).

The combination of hypericin and omeprazole can be seen in Figure 3. Because of the high cytotoxic effect of hypericin at $10^{-5}$ mol.l$^{-1}$ (less than 5 % of cells survived) we decided to use the $10^{-6}$ mol.l$^{-1}$ concentration in combination with omeprazole. If compared with hypericin alone, significant differences were found in response to all omeprazole concentrations used. They significantly ($P<0.001$) increased the phototoxicity of hypericin at $10^{-6}$ mol.l$^{-1}$.

The effect of DMA on HL-60 cell survival was tested under the same conditions as the effect of omeprazole. The cytotoxic effect of DMA alone was very high and light-independent in the concentration $10^{-5}$ mol.l$^{-1}$ (data not shown). No significant photoactivated cytotoxic effect was observed for lower concentrations of DMA ($10^{-6}$ to $10^{-10}$ mol.l$^{-1}$). The addition of DMA ($10^{-6}$ to $10^{-10}$ mol.l$^{-1}$) to $10^{-6}$ mol.l$^{-1}$ hypericin significantly ($P<0.001$) potentiated the cytotoxic effect of the latter substance (Fig. 4).

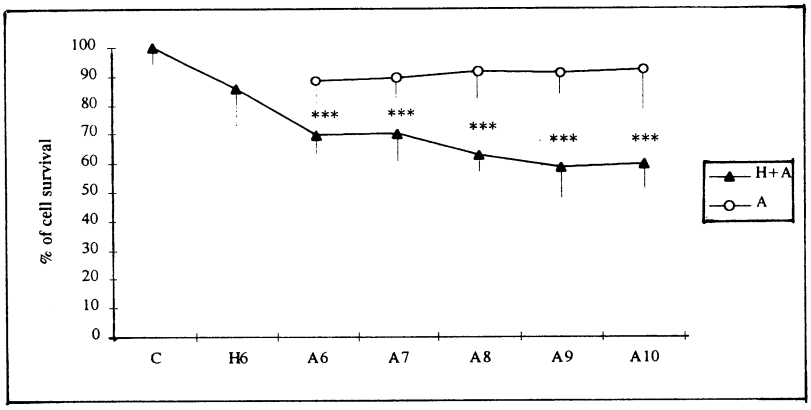



**Fig. 4.** *The effect of DMA alone (A) and in combination with hypericin (H+A) on cell survival. C – control cells without any treatment; H6 – cells treated with hypericin alone at $10^{-6}$ mol.l$^{-1}$; A6-A10 – DMA at $10^{-6}$ to $10^{-10}$ mol.l$^{-1}$ in combination with hypericin at $10^{-6}$ mol.l$^{-1}$. Significant differences versus hypericin alone are expressed as ***$P<0.001$.*

## Discussion

Our first experiments with hypericin clearly revealed the potentiating effect of light on its cytotoxicity. When comparing light and dark conditions, we observed significant differences in the cell lethality induced by hypericin in concentrations $10^{-5}$, $10^{-6}$ and $10^{-7}$mol.l$^{-1}$. The percentage of surviving cells fell to approximately 80 % in two concentrations ($10^{-6}$ and $10^{-7}$ mol.l$^{-1}$) and practically to zero in the concentration of hypericin at $10^{-5}$mol.l$^{-1}$. In some experiments, the activity of photoactivated hypericin was higher than the value obtained in our experiments. Its antiproliferative activity was found to be in the nanomolar range in some human tumor cell lines (Vandenbogaerde *et al.* 1998). The difference could be explained by the use of much lower intensity of illumination in our experiments (0.4 J/cm$^2$) as compared to 4 J/cm$^2$ in the above mentioned paper. Another possibility is the use of different cell lines (A431, HeLa and MCF7 in the cited article, HL-60 in our experiments) because photoactivated hypericin exerted a differential cytotoxic effect on various cell lines (Vandenbogaerde *et al.* 1997).

Because the aim of our studies was to evaluate the possible potentiating effect of cytoplasm acidifying agents in the cytotoxic mechanism of action of hypericin, we decided to combine its submaximal effective dose with several doses of omeprazole ($H^+K^+$-ATPase inhibitor and non-specific vacuolar $H^+$-ATPase inhibitor) and DMA (specific $Na^+/H^+$ exchanger inhibitor in the cell membrane).

Recently, it has been shown that besides the inhibitory effect of omeprazole on $H^+K^+$-ATPase it also inhibits the vacuolar $H^+$-ATPase of the turtle bladder (Graber and Devine 1993). Furthermore, although the major proton transport of osteoclasts is mediated by the vacuolar-type $H^+$-ATPase which is different from the gastric $H^+K^+$-ATPase. *In vitro* studies have demonstrated that omeprazole inhibits bone resorption. In addition, urinary excretion of hydroxyproline and calcium decreases after omeprazole treatment (Mizunashi *et al.* 1993). Furthermore, the vacuolar-type of $H^+$-ATPase from adrenal chromaffin granules has also been found to be sensitive to omeprazole (Moriyama *et al.* 1993). Finally, it was demonstrated (Fitton and Wiseman 1996) that a newer $H^+K^+$-ATPase inhibitor, pantoprazole, is

less active *in vitro* against the vacuolar $H^+$-ATPase responsible for lysosomal acidification ($IC_{50}$=194 vs 75 $\mu mol.l^{-1}$) than omeprazole.

One of the best characterized cation/$H^+$ exchangers is the $Na^+/H^+$ exchanger. There are several isoforms of this exchanger, some of which are specifically inhibited by the loop diuretic, amiloride. This exchanger is the main $H^+$-extruding mechanism in response to an intracellular acid load in a bicarbonate free solution.

A growing amount of evidence is accumulating that intracellular pH drop is an early apoptotic event. Cytoplasmic acidification is associated with the apoptotic process in retinamide-mediated apoptosis of human promyelocytic leukemia cells (Angoli *et al.* 1996), in the ara-C and daunorubicin-incubated cells Molt4, CEM and HL-60 (Neubauer *et al.* 1989).

It seems that hypericin exerts multiple modes of light-induced biological activity. Oxygen-dependent mechanisms of hypericin activity have been proposed by Thomas and Pardini (1992). Petrich and coworkers had reported that hypericin is also active under hypoxic conditions and had speculated that this mode of action involved excited-state proton transfer which finally leads to proton ejection into the hypericin environment and consequently to an acidification of cellular compartments (Fehr *et al.* 1994, 1995b). Such light-dose dependent local cellular pH drop were observed for 3T3 mouse fibroblasts in the presence of hypericin illuminated with 514 nm wavelength (Sureau *et al.* 1996).

All available information led us to use omeprazole for testing the hypothetical pH decreasing effect of hypericin in its cytotoxic effect on the neoplastic leukemic cell line HL-60. If hypericin decreases intracellular pH, omeprazole should increase the cytotoxic effect of hypericin by a partial block of the transport of hydrogen ions into intracellular organelles (e.g. lysosomes). Our experiments have shown that in the HL-60 cell line the effect of hypericin at $10^{-6}$ $mol.l^{-1}$ was significantly potentiated by omeprazole. The only disturbing event was the large variations of the means from different experiments, which is shown for omeprazole in Figure 3. These variations could be explained by the weak specificity of omeprazole for the vacuolar type of $H^+$-ATPase and its preferential effect on $H^+K^+$-ATPase.

This was also the reason why we tested a more specific modulator of intracellular pH and why we decided to test DMA as a highly specific inhibitor of $Na^+/H^+$ exchanger in the cell membrane. The effect of DMA was less pronounced than that of omeprazole, but it also significantly potentiated the cytotoxic effect of hypericin. However, the variations between the results in different experiments were less pronounced as compared to omeprazole. The results, which were more uniform and regular, support the hypothesis of Petrich and coworkers that excited-state proton transfer could play a role in the biological activity of hypericin. Our observation is also supported by the recent experiments of Weller *et al.* (1997) with LN-229 and T98G glioma cells that the pre-exposition of these cell lines to free radical scavengers such as N-acetylcysteine, superoxide dismutase and N-t-butyl-phenyl-nitrone had no protective effect and failed to affect the cytotoxicity induced by hypericin. Thus, free radicals do not seem to play a major role in the biological effect of hypericin. Moreover, both omeprazole and DMA have been shown to exert a significant potentiating effect to hypericin-induced cytotoxicity in the HL-60 cell line.

In conclusion, the data shown here suggest that the potential cytotoxic mechanism of the action of hypericin is at least partly mediated by reducing the intracellular pH. This possibility is supported by the finding that omeprazole as well as amiloride potentiate this effect.

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