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SHORT COMMUNICATION

Modulation of the Phototoxic Effect of Hypericin in Human Leukemia CEM Cell Line by N-Ethylmaleimide, Amiloride and Omeprazole

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Summary

Hypericin is a photosensitizing plant pigment from *Hypericum perforatum* with multiple modes of light-induced biological activities due to production of singlet oxygen and/or excited-state proton transfer with consequent pH drop in the hypericin environment. In the present work, we studied the effects of three inhibitors of crucial mechanisms responsible for intracellular pH (pH_i) regulation on hypericin phototoxicity: N-ethylmaleimide (NEM), an inhibitor of H^+-ATPase, 5'-(N,N-dimethyl)-amiloride (DMA), an inhibitor of Na^+/H^+ exchanger, and omeprazole (OME), an inhibitor of H^+K^+-ATPase. Our experiments show that the effect of hypericin at $1x10^{-5}$ and $1x10^{-6}$ $mol.l^{-1}$ was significantly potentiated by NEM ($1x10^{-7}$-$1x10^{-9}$ $mol.l^{-1}$) and DMA ($1x10^{-6}$ and $1x10^{-7}$ $mol.l^{-1}$) in leukemic CEM cell line. On the other hand, OME had no significant effect on hypericin cytotoxicity. Our results support the hypothesis that the excited-state proton transfer and the consequent acidification of hypericin environment could play a role in the biological activity of hypericin.

Key words

Hypericin • Phototoxicity • N-ethylmaleimide • Amiloride • Omeprazole • CEM cells

Hypericin is a naturally occurring pigment found in plants of *Hypericum* genus. This polycyclic quinone displays various biological effects including phototoxicity against a number of malignancies. It has been shown that exposure of hypericin to light induces a transfer of energy to the nearby oxygen molecules producing singlet oxygen which by itself is very toxic. However, later experiments revealed that hypericin was toxic even in situations when no oxygen was available (Fehr *et al.* 1994). Further studies suggested that another light-driven chemical process called "proton transfer reaction" might be responsible for the toxic effect of hypericin. Proton transfer reactions occur when proton moves for a short distance between neighboring oxygen atoms of a molecule. Based on these results, it might be presumed that the toxic action of hypericin should be potentiated by a decrease of pH in its environment.

On the basis of these informations we decided to test the influence of three compounds which are able to block different mechanisms responsible for pH_i control,

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NEM, DMA and OME, on the cytotoxic effect of hypericin. NEM could influence pH_i by a wider range of mechanisms. Besides others it acts as an inhibitor of V-type H^+-ATPases which were found in various membranous systems of eukaryotic cells. These ATPases are believed to play a significant role in the maintenance of the acidic environment in the organelles (Fuchs *et al.* 1989). Furthermore, there is an evidence for the presence of H^+-ATPases in the plasma membranes of tumor cells (Stone *et al.* 1989). Another substance with a more specific effect on intracellular pH is DMA. This agent acts as a specific inhibitor of membrane-associated Na^+/H^+ exchanger which responds to cellular acidification by extruding the H^+ ion in exchange for the influx of Na^+ (Madshus 1998). OME is a prodrug targeting H^+/K^+-ATPase of gastric parietal cells. Moreover, omeprazole is supposed to inhibit the vacuolar type H^+-ATPase (Mattsson *et al.* 1991).

Human acute lymphoblastic T-cell leukemia (CEM) cell line was used for all experiments. Cells were maintained in 1640 RPMI medium (Gibco, UK) containing 10 % fetal calf serum (FCS), 25 mg/100 ml glutamine and penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively). Hypericin (Carl Roth, Germany) was dissolved in DMSO (Sigma, Germany) and further diluted to final concentrations with 1640 RPMI medium. NEM (Sigma, Germany) was dissolved in ethanol and diluted to the final concentrations with RPMI. The substance of DMA (Sigma, Germany) was dissolved and diluted to the desired concentrations with RPMI. OME (Losec, Astra, Sweden) was freshly dissolved and adjusted to the desired concentrations with RPMI. The cell cultures were illuminated with a total light dose of 4 J/cm^2. CEM cells were incubated for 72 h with hypericin at concentrations 10^{-7}-10^{-5} mol.l^{-1} alone or in combination with NEM, DMA or OME at concentrations 10^{-9}-10^{-5} mol.l^{-1}, under light conditions. For the assessment of the cytotoxic effect of the tested agents the MTT method was used in all experiments. The values of absorbancy were expressed as percentage of cell survival in comparison with controls taken as 100 % survival. The statistical analysis of the data was performed using the mean values ± S.D. Statistical analysis of the result was carried out by Student´s t-test. The results with $p<0.05$ were taken as statistically significant.

The light-induced toxicity of hypericin on the CEM cell line was strictly dose-dependent, ranging from 48 to 95% decrease in total cell survival. Testing the effect of the whole concentration spectrum of NEM revealed its high toxicity at concentrations of 10^{-6} and 10^{-5} mol.l^{-1}. Lower concentrations did not influence cell survival (data not shown). With respect to these results, only non-toxic concentrations of NEM ($1x10^{-9}$, $1x10^{-8}$ and $1x10^{-7}$ mol.l^{-1}) were used in further experiments. NEM at concentration $1x10^{-7}$ mol.l^{-1} significantly potentiated the effect of hypericin at $1x10^{-5}$ and $1x10^{-6}$ mol.l^{-1} ($P<0.01$ and $P<0.001$), respectively. Lower concentration of NEM ($1x10^{-8}$ mol.l^{-1}) produced similar potentiation of hypericin toxicity. NEM at concentration $1x10^{-9}$ mol.l^{-1} significantly potentiated the toxicity of hypericin only at concentration $1x10^{-6}$ mol.l^{-1} ($P<0.001$) (Fig. 1).

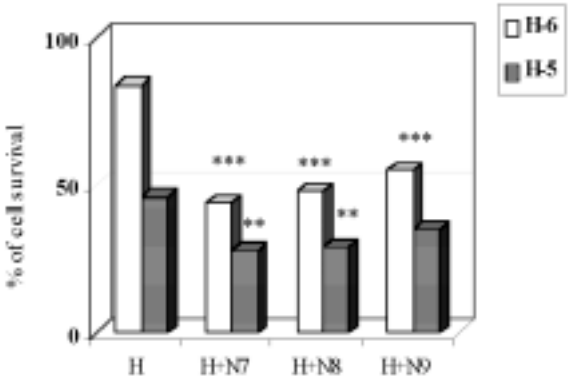

Fig. 1. *Effect of hypericin at concentrations $1x10^{-5}$ and $1x10^{-6}$ mol.l^{-1} (H-5 - H-6) on CEM cell survival if given alone (H) or in combination with NEM at concentration $1x10^{-7}$ mol.l^{-1} (H+N7), $1x10^{-8}$ mol.l^{-1} (H+N8) or $1x10^{-8}$ mol.l^{-1} (H+N9). The results are arithmetic means of 10 experiments. **P<0.01 vs. H-5, ***P<0.001 vs. H-6*

Hypericin at concentration $1x10^{-7}$ mol.l^{-1} did not produce a significant phototoxic effect either alone or in combination with NEM. Testing the effect of DMA alone revealed its toxicity only at the highest concentration ($1x10^{-5}$ mol.l^{-1}). Lower concentrations of DMA did not interfere with cell survival significantly (data not shown). In further experiments, we used only non-toxic concentrations of DMA ranging from $1x10^{-6}$ to $1x10^{-9}$ mol.l^{-1}. DMA at concentrations $1x10^{-6}$ and $1x10^{-7}$ mol.l^{-1} significantly potentiated the phototoxicity of the highest concentration of hypericin ($1x10^{-5}$ mol.l^{-1}) ($P<0.01$ and $P<0.05$), respectively (Fig. 2). The lower concentrations of hypericin were potentiated by DMA only non-

significantly (data not shown). OME itself was not toxic towards the CEM cell line. There was a slight increase in the cytotoxic effect of hypericin but statistical significance was not achieved for any concentration tested (data not shown).

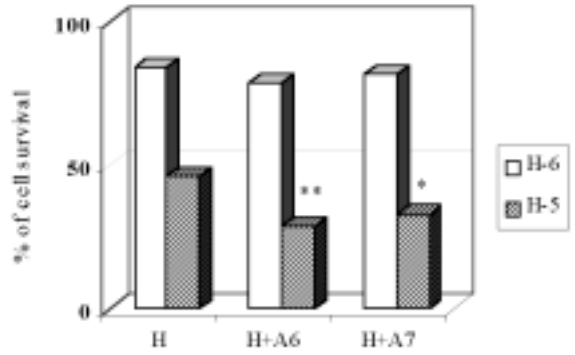

Fig. 2. *Effect of hypericin at concentrations $1x10^{-5}$ and $1x10^{-6}$ mol.l^{-1} (H-5 - H-6) on CEM cell survival if given alone (H) or in combination with DMA at concentration $1x10^{-6}$ mol.l^{-1} (H+A6) and $1x10^{-7}$ mol.l^{-1} (H+N7). The results are arithmetic means of 10 experiments. ** P<0.01 vs. H-5, * P<0.05 vs. H-5.*

Maintenance of pH_i within the physiological range is crucial for normal cell function due to the narrow pH optima of many intracellular processes (Madshus 1998). Among the mechanisms controlling pH_i an important role is played by Na^+/H^+ exchangers and Na^+-dependent and -independent HCO_3-transporters (Russell *et al.* 1983) as well as ATP-dependent transporters such as H^+-ATPases and H^+/K^+-ATPases (Martinez-Zaguilan *et al.* 1993). Recent studies documented that acidification of the cytoplasm is associated with apoptosis (Meisenholder *et al.* 1996). One of the supposed mechanisms of hypericin phototoxic action involves proton transfer with a consequent pH drop. According to the above-mentioned studies and also our previous work (Mirossay *et al.* 1999), we suggest that the compounds able to influence pH_i should potentiate phototoxic effect of hypericin. Inhibitors of crucial mechanisms responsible for pH_i regulation – NEM (an inhibitor of H^+-ATPase), DMA (an inhibitor of Na^+/H^+ exchanger), and OME (an inhibitor of H^+/K^+ ATPase) – were tested on hypericin-induced phototoxicity.

The experiments demonstrated that the cytotoxic effect of hypericin was significantly potentiated by NEM and DMA, indicating that decrease of pH_i may be involved in hypericin activity. This hypothesis is also supported by other recent reports, which documented that amiloride and NEM are both potent acidificators of the cell cytoplasma even if given alone (Zhang *et al.* 1996). Surprisingly, OME produced only a non-significant potentiating effect on hypericin cytotoxicity in the CEM cell line. Explanation of these different effects of omeprazole on hypericin phototoxicity in HL-60 cells (promyelocytic leukemia) and CEM cells (lymphoblastic T-cell leukemia) is only speculative but it may be due to a great variability and specialized physiological role of non-gastric H^+/K^+-ATPases in different cells and tissues as reported by Jaiser and Beggah (1999).

On the basis of these results we suggest that NEM and DMA potentiate the light-induced effect of hypericin probably due to the cytoplasmic acidification. Our results indirectly support the hypothesis that this mechanism which results from the excited state-proton transfer could be involved in the phototoxic effect of hypericin. However, the generally believed important role of oxygen and free radicals was also demonstrated in recent studies (Delaey *et al.* 2000, Miroššay *et al.* 2001) and we suggest that the biological activity of hypericin is due to a combination of both mechanisms.

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