

# Inhibition of the CD8<sup>+</sup> T cell-mediated cytotoxicity reaction by hypericin: potential for treatment of T cell-mediated diseases

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**Keywords:** apoptosis, cytotoxic T lymphocyte, cytotoxicity, hypericin, protein kinase C, tumor necrosis factor- $\alpha$

## Abstract

The cytotoxicity reaction of murine CD8 T lymphocytes has been found to be strongly inhibited by nanomolar concentrations of hypericin, a lipophilic dianthraquinone with photodynamic properties. Cytotoxic T lymphocyte (CTL)-induced target cell apoptosis, as well as exocytosis of cytolytic granules from these cells, were ablated by hypericin, administered at the onset of the reaction, without affecting CTL viability. The inhibition of cytolysis occurred without the light irradiation which is essential for photosensitization. The findings suggest that the action of hypericin targets the effector CTL; however, apoptosis induced in murine L-cells with recombinant tumor necrosis factor (TNF)- $\alpha$  was also prevented by hypericin. Since hypericin is a known inhibitor of protein kinase C, MAP kinase and at least one other tyrosine kinase, this inhibitory activity could play a role in the down-modulation of CTL-induced cytotoxicity. Furthermore, our studies show that the action of hypericin induces rapid dephosphorylation of phospholipids associated with low-density membranes in CTL, but not with membranes of the cytotoxic granules. The ability of hypericin to interfere with cytotoxicity may render it useful in the treatment of T cell-mediated diseases.

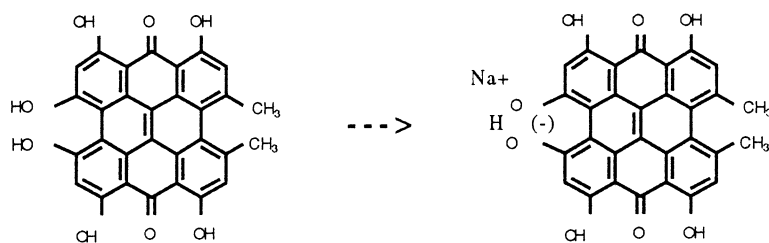
## Introduction

Perihydroxylated polycyclic quinones are the focus of increasing interest due to their broad range of biological activities. One compound in this group, hypericin (Fig. 1), is a photodynamic agent which we have initially found to be virucidal to retroviruses (1,2) as well as to other lipid enveloped viruses (3). Hypericin also acts as an irreversible light-dependent inhibitor of protein kinase C (PKC) (4) and MAP kinase (5), but has negligible effects on casein kinase I and II or on protein kinase A (5). Inhibition of epidermal growth factor receptor tyrosine kinase has also been suggested, affecting the enzyme in its membrane-associated native form, but not when solubilized (6). These inhibitory activities of cell proliferation signal transduction pathways prompted the clinical evaluation of hypericin as an antitumoral agent inhibiting the growth of malignant glioma (7,8) and pituitary adenoma tumors (9). The two tumors rely on PKC signaling for cell proliferation (7,8). Recently, the phototoxic properties of hypericin have also been addressed and found to be useful in the photodynamic therapy of tumors, in treatments coupled with

light irradiation (10). Clinical efficacy studies are currently ongoing to evaluate this photosensitizer against cutaneous T cell lymphoma.

The physico-chemical properties of hypericin are complex. In the presence of light hypericin generates singlet oxygen (11,12), free radicals (12,13) and under some circumstances also semiquinones (14). In addition, effects such as growth inhibition of malignant glioma cells in culture are independent of light (7); the virucidal activity of hypericin, while strongly enhanced by light (15), has also been documented in the dark against murine cytomegalovirus (16). The dark effects do not necessarily reflect a different mode of action and appear to be due to a low red/ox potential, enabling hypericin to act as an electron scavenger from physiological transfer reactions (17).

Here we report that hypericin effectively inhibits the cytotoxicity reaction of sensitized cytotoxic T lymphocytes (CTL) *in vitro*. This effect was associated with dephosphorylation of phospholipids in the low-density membrane fractions of CTL.



**Fig. 1.** The structure of the free acid form of hypericin (left) and its more soluble monosodium ion pair form (right).

We suggest that hypericin may be useful in the treatment of autoimmune diseases such as psoriasis and possibly also various forms of organ transplant rejection, in the pathogenesis of which cytotoxic effects of T cells play a role.

## Methods

### Preparation of hypericin

Hypericin (10,11-dimethyl-1,3,4,6,8,13-hexahydroxy-naphthodianthrone) was synthesized by self-condensation of emodin anthrone (18). Emodin anthrone (Societa Inverni della Befra, Milan, Italy) was dissolved in pyridine and heated with piperidine, pyridine-*N*-oxide and catalytic amounts of  $\text{FeSO}_4$  (Aldrich Chemicals), resulting in the formation of protohypericin. Photoactivated ring closing was obtained by irradiation of protohypericin with visible light to yield free hypericin, which was crystallized from pyridine, resulting in a hypericin-pyridine complex. This complex was heated to  $160^\circ\text{C}$  for 2 h in high vacuum. The resulting free hypericin was dissolved in methanol and converted to a monosodium salt by adding aqueous  $\text{NaHCO}_3$ , precipitation with hexane and crystallization from methanol. Hypericin was purified to 98.7% by chromatographies on silica gel (Merck 60; 70-230 mesh) eluted with methanol:EtOAc 2:1 and 1% aqueous  $\text{NaH}_2\text{PO}_4$ . For the bioassays hypericin was dissolved to 4 mM in 70% aqueous ethanol. Subsequent dilutions were made in cell culture media limiting the final EtOH concentration to 1%.

### Chemicals, isotopes, reagents and antisera

Recombinant tumor necrosis factor (TNF)- $\alpha$  was a gift from Dr Joseph Lotem (Department of Molecular Genetics, Weizmann Institute, Rehovot Israel).  $\text{Na}^{51}\text{CrO}_4$  and  $\text{Na}_2\text{H}^{32}\text{PO}_4$  from New England Nuclear Du-Pont (Wilmington, DE). Phytohemagglutinin (PHA)-P was purchased from Sigma (St Louis, MO).

### Cell lines

Murine P815 cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 100 mM L-glutamine, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. L-cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FCS, glutamine and antibiotics. All cell lines were cultured in a humidified 5%  $\text{CO}_2/95\%$  air atmosphere at  $37^\circ\text{C}$ .

### Cell viability

Cell viability was monitored by the MTT assay, which measures reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazol-

ium bromide to formazan in mitochondria of viable cells (19). MTT was added to cells cultured in 96-well plates (in triplicates) 48 h after induction of apoptosis. The cultures were incubated for 4 h at  $37^\circ\text{C}$  and analyzed in an ELISA reader at 560 nm. Corrections for non-specific optical absorption by MTT were made in medium with or without hypericin at each corresponding dose level. The optical density of formazan generated by untreated cell cultures ( $\text{OD}_{\text{control}}$ ) was defined as 1 MTT unit. The number of MTT units in culture samples undergoing treatments was calculated as the ratio ( $\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}/\text{OD}_{\text{control}}$ ).

### Development of CTL clones sensitized against class I or class II histocompatibility complex determinants

Two sets of CTL clones were developed. Anti-H-2<sup>d</sup> CTL generated in spleen and lymph node cells from C3H (H-2<sup>b</sup>) mice sensitized against (C3H $\times$ BALB/c) $F_1$ , (H-2<sup>b/d</sup>) stimulator cells; and anti-class II I-A<sup>q1</sup>-E<sup>q1</sup> CTL developed from B10.AQR cells sensitized against B10.T(6R) cells (H-2 K<sup>q1</sup>-A<sup>q1</sup>-E<sup>q1/d</sup>). From each group,  $4 \times 10^6$  responder cells were sensitized against  $2 \times 10^7$  X-irradiated (1500 rad) stimulator spleen cells for 5 days. Re-stimulations with  $5 \times 10^6$  freshly prepared irradiated spleen cells were at 5 day intervals and at day 15 the growth medium was supplemented with 100 U/ml of rIL-2 (Cetus, Norwalk, CT). On day 14 the cells were cloned by limiting dilution in 96-well microplates on  $5 \times 10^4$  irradiated stimulator feeder cells in medium DMEM, 15% fetal bovine serum, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. rIL-2 at 100 U/ml was added after 5 days and subsequently reduced to a maintenance dose of 75 U/ml. Isolated colonies were subcloned and expanded in 50 ml tissue culture flasks. Cloned cells were stained with fluorescein-labeled anti-CD4 and -CD8 antibodies (PharMingen, San Diego, CA), and analyzed by FACS. The different clones were screened for cytotoxic activity against  $^{51}\text{Cr}$ -labeled appropriate targets and the clones which were most cytotoxic were selected for experimentation.

### Cytotoxicity assays

The cytotoxic activities of the CD8<sup>+</sup> T cell clones were examined in 4 h  $^{51}\text{Cr}$ -release assays: C3H CTL against P815 leukemia cells (H-2<sup>d</sup>) targets and the other CTL set against PHA stimulated primary spleen cells (PHA blasts) derived from the corresponding stimulator mice strains. Assays were run in triplicates in 96-well round-bottom microplates. From the time hypericin was added to the cultures the microplates were maintained in relative darkness (hood lights off), to

prevent light-induced phototoxicity due to the photodynamic properties of hypericin. The total exposure to light was monitored carefully and kept  $<0.05 \text{ J/cm}^2$ . Experiments were repeated at least 3 times. Percent specific cytotoxicity was calculated according to the formula: (sample  $^{51}\text{Cr}$  release – spontaneous release)/(maximal release – spontaneous release)  $\times 100$ .

#### *Exocytosis of granules from CTL*

The specific discharge of *N*- $\alpha$ -benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterase activity into the reaction medium of cytotoxicity assays, was used to monitor exocytosis of granzyme-containing granules from CTL. CTL-conditioned, cell-free reaction media were collected 2 h after initiation of cytotoxicity. Aliquots of 20  $\mu\text{l}$  were incubated with 180  $\mu\text{l}$  enzyme substrate, which consisted of 30  $\mu\text{l}$  of 20 mM *N*-benzyloxycarbonyl-L-lysine thiobenzyl ester (Calbiochem, San Diego CA), dissolved in 0.1 M Tris-HCl (pH 8), mixed shortly before the assay with 30  $\mu\text{l}$  of 20 mM DNTB (Pierce, Rockford, IL) in 3 ml Tris buffer. The assay was run for 30 min at room temperature (20) and substrate hydrolysis analyzed at 405 nm in an ELISA plate reader. Specific granule exocytosis was calculated as the difference between BLT esterase activity released into the reaction medium by CTL activated with P815 targets and BLT esterase activity released spontaneously by CTL. Only experiments in which spontaneous BLT release was  $<10\%$  were considered.

#### *Determination of percentage of apoptotic cells*

Percentage of apoptotic cells, characterized by nuclear fragmentation, chromatin condensation and DNA internucleosomal digestion, was determined microscopically using the TUNEL POD method of Boehringer Mannheim (Mannheim, Germany) (in which TdT-added terminal fluoresceinated nucleotide is visualized with peroxidase conjugated anti-fluorescein). One hundred cells in four preparations (total of 400 cells) were counted by two individuals, independently. The data are given as the mean and SD calculated per four events.

#### *Induction of phototoxicity*

Photodynamic toxicity is the damage inflicted upon target cells by a photosensitizer in the presence of light. Photosensitization generates singlet oxygen and, in the case of hypericin, additional reactive oxygen species such as free radicals. Light irradiation was performed from two parallel 40 W fluorescent tubes placed at a fixed distance of 16 cm and measured to emit an incidence of 4 mW/cm of polychromatic white light. Light intensities were quantitated using the IL 1350 radiometer/photometer from International Light (Newburyport, MA).

#### *Radiolabeling and subcellular fractionation of CTL*

CTL ( $2 \times 10^7$ ) were washed twice with phosphate-deficient DMEM (Gibco, Grand Island, NY) and incubated for phosphate starvation in 2 ml of this medium supplemented with 2% FCS (Hyclone, Logan, UT) for 1 h. The cells were spiked with 50  $\mu\text{Ci}$   $\text{Na}_2\text{H}^{32}\text{PO}_4$  and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  for 90 min. Labeling was terminated with excess PBS and free  $\text{Na}_2\text{H}^{32}\text{PO}_4$  was removed by washing 3 times with PBS. The

cells were dispersed  $4 \times 10^6/\text{tube}$  in normal DMEM supplemented with 10% FCS and treated with 10  $\mu\text{M}$  hypericin for different time intervals. Following incubation the cells were disrupted in hypotonic lysis buffer (5 mM Tris pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM EGTA and 1 mM dithiothreitol) and homogenized in a loosely fitted Dounce homogenizer. Complete Protease Inhibitor Cocktail (40  $\mu\text{l}/\text{ml}$ ; Boehringer Mannheim) was added, the cell lysates brought to 30% in sucrose, loaded onto discontinuous 40, 45 and 50% sucrose gradients, and spun at 100,000  $g$  for 30 min. Cytosol and all interfaces were collected, the sucrose removed by dialysis against Buffer A (10 mM Tris, pH 7.5, 5 mM  $\text{MgCl}_2$  and 1 mM EGTA), and the various separated membrane fractions pelleted at 20,000  $g$  for 30 min. All fractions were solubilized in buffer A containing 1% Triton X-100 and separated on 14% SDS-PAGE. The gels were dried and developed for autoradiography.

## Results

#### *The effect of hypericin on CTL-mediated target cell apoptosis*

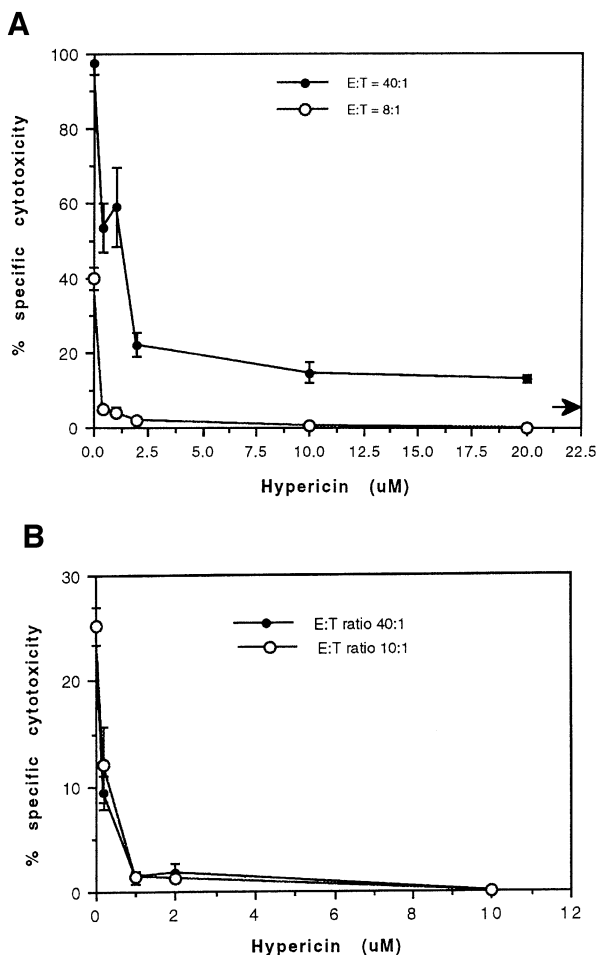
The two cytotoxic CD8 T cell clones which were developed, against H-2<sup>d</sup>, class I and MHC class II I-A<sup>qI</sup>-E<sup>q</sup> antigens were analyzed for the effects of hypericin on their ability to induce target cell apoptosis. Different doses of hypericin were added at the beginning of the cytotoxicity reactions with all subsequent steps performed under controlled lighting conditions (see Methods). Figure 2(A) shows the effect of hypericin on 4 h  $^{51}\text{Cr}$ -release assays of C3H CTL reacted against P815 targets. Figure 2(B) relates to the effect of hypericin on the activity of B10.AQR CTL reacted against class II I-A<sup>qI</sup>-E<sup>q</sup>-expressing PHA blasts in the presence of hypericin. Hypericin was found to inhibit target cell apoptosis by sensitized CTL in both systems. Doses of 0.4 and 1  $\mu\text{M}$  hypericin effectively inhibited 50–60 and  $\leq 90\%$  of the cytotoxic activity of the corresponding untreated cells respectively in most analyses. The strong inhibitory activity of target cell apoptosis by hypericin appears, quite likely, to be a light-independent process.

#### *The effect of hypericin on cytotoxic granule exocytosis from CTL in a cytotoxicity reaction*

The release of granules from CTL, that contain granzymes and pore-forming perforins (21,22), is another functional arm of the cytotoxicity reaction. We examined whether hypericin also affected granule exocytosis from effector CTL. CD8 cells sensitized against H-2<sup>d</sup> were stimulated with P815 targets to induce granule exocytosis. The cell-free reaction media were collected after 2 h and their granule contents analyzed in the BLT esterase assay. The results are shown in Fig. 3. A hypericin dose-dependent inhibition of granule exocytosis from CTL was observed that was linear up to a hypericin concentration of 1  $\mu\text{M}$  and reached baseline levels at 2  $\mu\text{M}$ .

#### *Hypericin toxicity to CTL and the role of light in the inhibition of CTL-mediated apoptosis*

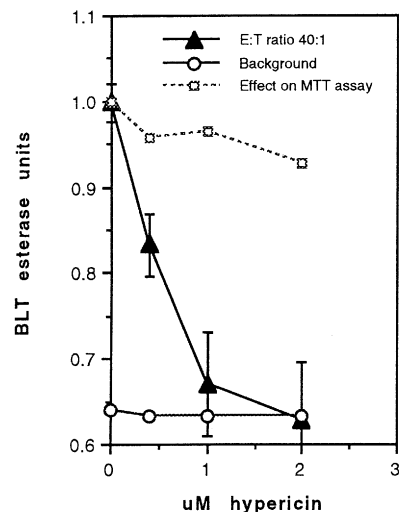
The possibility that the inhibition of cytotoxicity by hypericin stems from toxicity to CTL has also been addressed. The T cell clones were exposed to different concentrations of hypericin for 4 h under the same conditions in which  $^{51}\text{Cr}$



**Fig. 2.** The effects of hypericin on murine CTL-mediated cytotoxicity. Effector CTL,  $2 \times 10^5$ /well, plated in 96-well round-bottom plates, were treated with 0, 0.4, 2, 5 and 20  $\mu\text{M}$  hypericin, in triplicates under dark conditions (hood lights off and total exposure to light kept  $< 0.05 \text{ J/cm}^2$ ).  $^{51}\text{Cr}$ -labeled targets were then added and a 4 h release assay then initiated. Each experiment was repeated 3–5 times using two E:T cell ratios: 40:1 and 10:1. Target cells treated with the corresponding doses of hypericin served as spontaneous release controls and were used to subtract background  $^{51}\text{Cr}$  release. Two different systems were studied. (A) An anti-MHC H-2<sup>d</sup> reaction generated by sensitized C3H (H-2<sup>b</sup>) CTL reacted with  $^{51}\text{Cr}$ -labeled P815, H-2<sup>d</sup> targets. Arrow at the lower right depicts level of cytotoxicity against class I incompetent B10.AQR spleen cells (non-specific cytotoxicity). (B) Anti-MHC, class II I-A<sup>q</sup>I-E<sup>q</sup> determinants by B10.AQR CTL reacted with PHA-stimulated, primary B10.T(6R) target cells.

release from target cells was inhibited. Lighting was controlled keeping hood lights shut off. Cell viability was analyzed after 4 h by Trypan blue exclusion. CTL viability was unimpaired by the treatment with hypericin at doses which prevented target cell apoptosis and granule exocytosis (data not shown). Toxicity to CTL appears unlikely to be the cause for the observed inhibition of CTL-mediated target cell apoptosis by hypericin. Figure 4 shows that inhibition of both arms of the cytotoxicity reaction,  $^{51}\text{Cr}$  release and granule exocytosis results from the action of hypericin on CTL and not on the target cells.

Since many of the biological activities of hypericin are

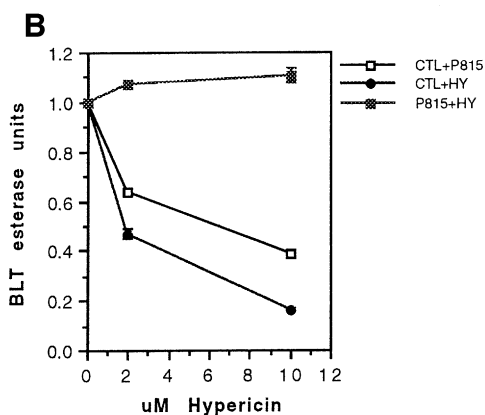
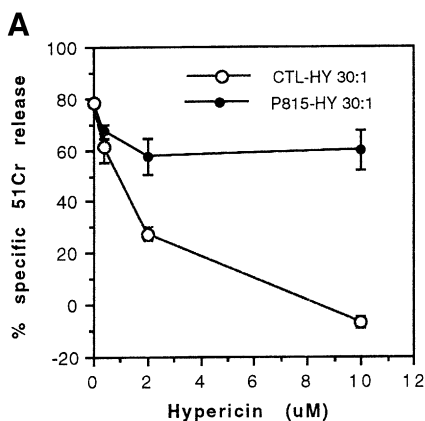


**Fig. 3.** The effect of hypericin on granule exocytosis from cytotoxic cells. CTL clones sensitized against H-2<sup>d</sup> were stimulated with P815 cells (in triplicates). Hypericin at concentrations of 0, 0.4, 1 and 2  $\mu\text{M}$  was added, the cultures spun gently at 500  $g$  for 2 min, and incubated at 37°C, 5%  $\text{CO}_2$  for 2 h. The cell-free reaction media were collected and assayed for BLT esterase activity. Another set of controls included supernatants from samples assayed for cytotoxicity in the absence of hypericin to which the drug was added prior to the BLT esterase assay to monitor for possible interference with the assay by hypericin. Open circles refer to baseline levels of BLT esterase released from unstimulated, sensitized CD8 cells plated in the absence of target cells. Results are given  $\pm$  SD.

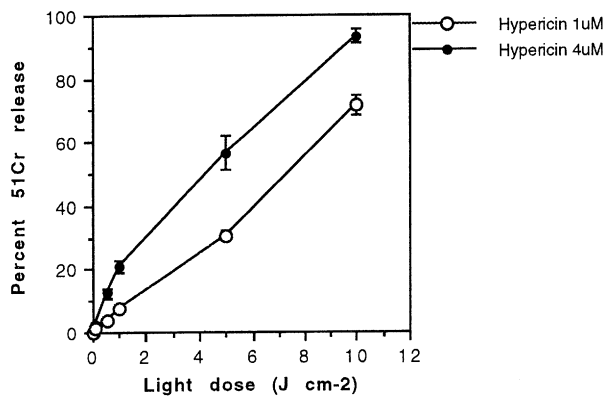
mediated or enhanced by light, the possible role of light in the arrest of CTL-induced cytotoxicity by hypericin was examined. Direct analyses of the effects of hypericin in conjunction with light on cytotoxicity were hampered by light-mediated increases in the levels of spontaneous  $^{51}\text{Cr}$  release from P815 cells. This complicated the distinction between specific  $^{51}\text{Cr}$  release and light-induced phototoxicity. The problem was circumvented by evaluating the effects of hypericin directly on  $^{51}\text{Cr}$ -labeled P815 cells maintained under conditions similar to those in cytotoxicity assays. The cells were photosensitized with 1 and 4  $\mu\text{M}$  hypericin, irradiated with 0.1, 0.5, 2 and 10  $\text{J/cm}^2$  of white fluorescent light, and incubated at 37°C for 5 h.  $^{51}\text{Cr}$  release was then determined, showing that the two tested doses of hypericin became phototoxic to P815 cells at light doses that exceeded 0.5  $\text{J/cm}^2$  (Fig. 5). The total dose of ambient light during benchwork was maintained  $\leq 0.03 \text{ J/cm}^2$ .

#### *The effects of hypericin on target cell apoptosis induced by TNF- $\alpha$ receptor ligation*

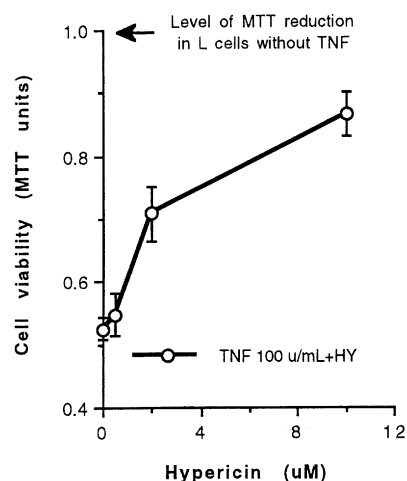
Hypericin interacts with cell membrane phospholipids (23) and can modulate some of their functions including transduction of signals generated at the cell surface (5,6). Since many of the apoptosis-inducing signals are initiated at the cell surface, the possible effects that treatment with hypericin can have on cells triggered to undergo apoptosis were studied using murine L-cells, which are highly susceptible to TNF- $\alpha$ . L-cells exposed to different concentrations of hypericin were then induced to undergo apoptosis with 100 U/ml TNF- $\alpha$ . Cell viability was analyzed after 48 h and compared to control



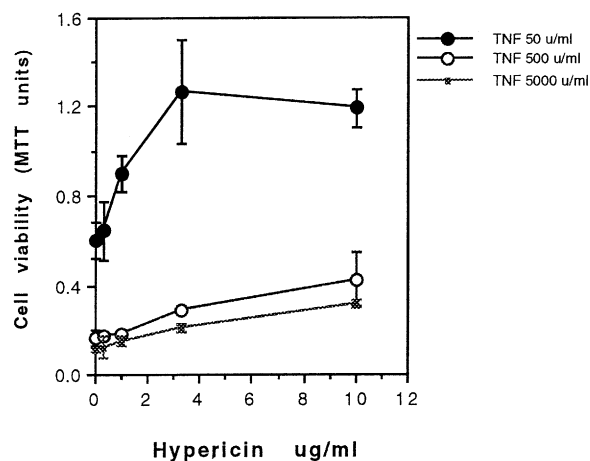
**Fig. 4.** The effect of differentially binding hypericin to the effector CTL, or to P815 target cells, on cytotoxicity. CTL or P815 cells were preincubated with hypericin for 30 min in complete medium at 37°C in the dark. Cell-free hypericin was removed by washing 3 times with medium. Cytotoxicity was analyzed at an E:T ratio of 30:1: (A) on <sup>51</sup>Cr release and (B) on granule exocytosis.



**Fig. 5.** The effect of light on isotope release from P815 cells. <sup>51</sup>Cr-labeled P815 cells were plated 10<sup>4</sup>/well in round-bottom 96-well microplates. Hypericin was added at a final concentration of 1 and 4 μM, and the plates exposed to white fluorescent light that emits at wavelengths between 380 and 700 nm at a fluence rate of 1.5 mW/cm<sup>2</sup>. The light dose was calculated from the exposure time. At each time point triplicate samples were transferred to another plate that was incubated at 37°C, 5% CO<sub>2</sub> for 4 h and assayed for isotope release.



**Fig. 6.** The effect of hypericin on TNF-α-induced apoptosis in murine L-cells. L-cells were plated 5×10<sup>4</sup>/well in 96-well plates. Hypericin, at doses of 0.65, 2, 6.5 and 20 μM, was administered 20 h after plating followed by 100 U/ml of TNF-α. The cells were cultured for an additional 48 h and their viability was then monitored by the MTT assay.



**Fig. 7.** Protection of murine L-cells by hypericin from apoptosis induced with different doses of TNF-α. Hypericin at doses of 0.65, 2, 6.5 and 20 μM was added to 5×10<sup>4</sup> L-cells/well, 20 h after plating. TNF-α was then applied at each of three doses: 50, 500 and 5000 U/ml. The cells were cultured at 37°C, 5% CO<sub>2</sub> for an additional 48 h and their viability was then assessed by the MTT assay. Data are averages of triplicate wells ± SD from a representative experiment of four that were performed.

cells not exposed to TNF-α, using the MTT assay. The results (Fig. 6) show an effective hypericin dose-dependent protection of L-cells from TNF-α-induced apoptosis. Cell viability was preserved at near control levels by 10 μM hypericin. The potency of the protective effect was estimated using different TNF-α concentrations and evaluating L-cells protection from apoptosis by hypericin. The results (Fig. 7) show that protection was most effective at the lowest dose level of 50 U/ml of TNF-α; however, some increases in cell viability, although of a lesser magnitude, occurred at the higher TNF-α doses of 500 and 5000 U/ml, in a hypericin

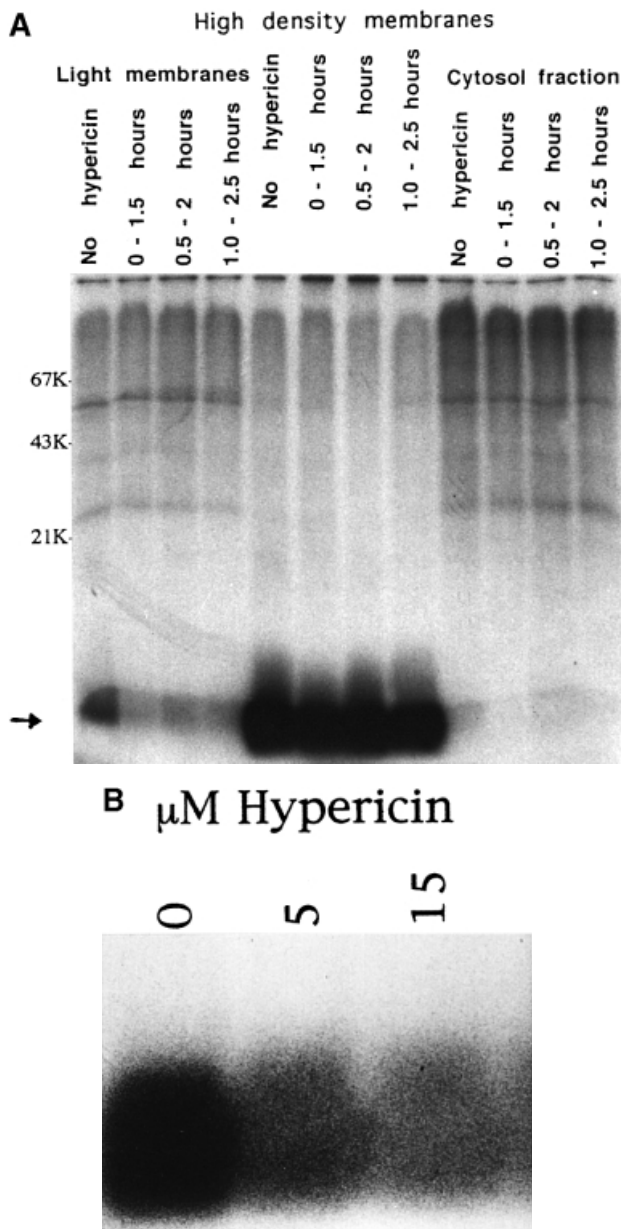
dose-related manner. The TNF- $\alpha$ -activated apoptotic pathway appears to also be affected by hypericin, unrelated to the inhibitory effects of this molecule on CTL.

#### Dephosphorylation of phospholipids on CTL low-density membranes by hypericin

Numerous studies that examined possible subcellular targets for the action of hypericin on CTL focused on possible effects of this molecule on protein phosphorylation or dephosphorylation. These were driven by the recognition that, on one hand, hypericin acts as inhibitor of numerous classes of cellular kinases and, on the other, that photosensitization activates phospholipases in mouse lymphoma cells (24). The most noticeable effects of hypericin were on phospholipids of the low-density membrane fraction. CTL prelabeled with [ $^{32}$ P]orthophosphate were treated with 10  $\mu$ M hypericin for 0.5, 1 and 1.5 h. Unincorporated isotope was removed, the cells lysed by hypotonic shock and fractionated on sucrose-density gradients into cytosol, and low-density membranes (30/40% interphase which contains the endoplasmic reticulum and surface membranes) and higher-density membranes recovered from the 40/45 and 45/50% interphases (25), as described in Methods. The different isolated fractions were separated on SDS-PAGE and developed for autoradiography. Figure 8(a), shows that the treatment with hypericin induced a rapid dephosphorylation of phospholipids in the low-density membrane fractions of CTL which migrated close to the gel front. The dephosphorylation occurred within short incubation periods with 10  $\mu$ M hypericin. The phospholipid nature of these bands was confirmed by extraction with chloroform:methanol 1:2, which eliminated the lipid bands in subsequent runs, and by thin layer chromatography of the solvent moiety on silica gel plates developed with  $\text{CHCl}_3$ :MeOH:NH $_4$ OH:H $_2$ O (26:15:3:1 v/v) (data not shown). Similar, low-density membrane dephosphorylation patterns were observed following 3 h treatments with 5 and 15  $\mu$ M hypericin (Fig. 8b). The bands were excised from the gels and the radioactivity determined in a  $\gamma$ -counter showing 64 and 76% inhibition of  $^{32}$ P incorporated into the phospholipid band by treatments with 5 and 15  $\mu$ M hypericin respectively. There was no evidence for effects on higher-density membrane fractions which harbored BLT hydrolytic activity (Fig. 9), suggesting that the dephosphorylating activity of hypericin appeared not to have affected the cytotoxic granules directly. However, the compound caused some decline in BLT enzymatic activity.

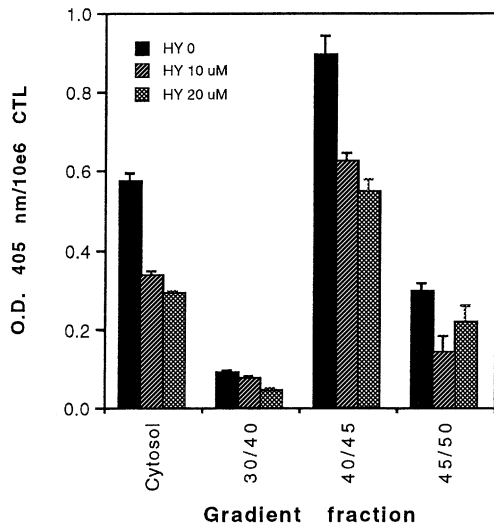
#### Discussion

The studies which are described depict hypericin as a potent inhibitor of T cell-mediated cytotoxicity by murine CD8 $^+$  CTL. The action is rapid and it was sufficient to administer hypericin at the beginning of cytotoxicity in order to effectively inhibit the reaction. It may be noteworthy that hypericin also inhibits human NK cell-mediated K-562 target cell lysis in a similar manner (data not shown and will be discussed elsewhere). CTL appear to be the affected cells as evident from experiments in which CD8 T cells or target cells were each treated separately with hypericin (Fig. 4). Association of the molecule with CTL inhibited cytotoxicity at two levels:  $^{51}\text{Cr}$  isotope



**Fig. 8.** Dephosphorylation of CTL low-density membrane phospholipids after treatment with hypericin. [ $^{32}$ P]orthophosphate-labeled CTL were treated with 10  $\mu$ M hypericin for 30 min at 37°C (A). The compounds were removed with PBS and samples collected 0.5, 1 and 1.5 h after the treatment. (B) The cells were incubated with 0, 5 or 15  $\mu$ M hypericin and incubated at 37°C for 2 h. The cells were then lysed by a hypotonic shock, and fractionated on discontinuous 30/40, 40/45 and 45/50% sucrose gradients. The cytosol and membrane fractions were solubilized in buffer A containing 1% Triton X-100, separated on 14% SDS-PAGE, dried and developed for autoradiography. Lipids are shown by arrow.

release from target cells was suppressed and granule exocytosis from T cells was ablated in a hypericin dose-dependent manner. The effect could result either from the action of hypericin on each of the two pathways or, alternatively, by inhibiting the activation of the lytic pathway upstream to the divergence into the two arms of the response.



**Fig. 9.** Distribution of BLT esterase activity between the cytosol and the different subcellular membrane fractions of  $10^7$  CTL treated with 0, 10 or 20  $\mu\text{M}$  hypericin for 30 min at  $37^\circ\text{C}$ . The different fractions were obtained after separation on discontinuous sucrose-density gradients.

The complexity of the cytotoxicity reaction offers many target sites for inhibition by hypericin: adhesion molecules (26,27) and lymphocyte function-associated antigens (28,29) are involved in effector–target cell interactions, with engagement of the TCR and activation of the lytic pathway. The outcome is presentation of Fas ligand on CTL followed by secretion of granules containing pore-forming perforin, proteolytic granzymes (21,30–31) and the cytotoxic molecule granulysin (32). Potential targets for CTL lytic pathway deactivation by hypericin may be phosphoproteins which participate in transduction of signals that culminate in lytic pathway activation and granule exocytosis. Their phosphorylation can be inhibited by hypericin, a well-documented potent inhibitor of PKC (4,5), epidermal growth factor receptor tyrosine kinase (6) and MAP kinase (4). In  $\text{CD8}^+$  CTL clones, Fas ligand induction by engagement of TCR is inhibited by herbimycin and by the more general protein tyrosine kinase (PTK) inhibitor genistein and ionomycin (33). In NK cells, protein tyrosine phosphorylation has been suggested to be required for cytotoxicity and killing is also inhibited with the PTK inhibitor herbimycin (34). Numerous tyrosine kinases,  $p56^{\text{Lck}}$ ,  $p59^{\text{fyn}}$  and the *syk* family kinase ZAP-70, are phosphorylated during NK cell activation (35). Their role in the cytolytic responses is unclear, however, since knockout mice deficient in these genes exhibit unimpaired cytolytic responses. It appears unlikely that their inhibition by hypericin accounts for the prevention of cytolysis.

The most noticeable change that we observed in CTL after hypericin treatment is the rapid phospholipid dephosphorylation that is confined to the low-density membrane fractions and becomes evident within 0.5–1.5 h after addition of hypericin to CTL. This dephosphorylation suggests that the treatment with hypericin activates phospholipases in CTL. Phospholipase C activation has been demonstrated in mouse L5178 lymphoma cells following photosensitization with another sensitizer—aluminum phthalocyanine (24). It was associated with break-

down of membrane phosphoinositides and increased arachidonic acid release. Phospholipase  $A_2$  has also been implicated in this phospholipid breakdown (24). Phospholipase D1, which localizes in resting cells to secretory granules and lysosomes, has been shown to translocate to the plasma membrane upon cellular stimulation (36). Modulation of phospholipase D1 could also be associated with the observed inhibition of granule exocytosis by hypericin. Although phospholipase activation by the phthalocyanine derivative was light dependent and the dephosphorylation of CTL low-density membrane fractions by hypericin, described here, occurred under minimal lighting conditions, the underlying mechanisms may be similar. A role for red/ox reactions cannot be entirely excluded. Hypericin is known to elicit photodynamic reactions in the dark, most likely due to its relatively low red/ox potential which, as mentioned above, enables electron scavenging for its excitation (17).

While treatment of target cells with hypericin in CTL-mediated cytotoxicity showed negligible effect on their levels of apoptosis and considerable evidence points to the effector cell as the site at which hypericin inhibits cytotoxicity, some phases of the apoptotic cascade in dying cells appear to also be affected. For example, hypericin was noted to effectively protect murine L-cells from apoptosis induced by quite high doses of  $\text{TNF-}\alpha$ , maintaining L-cells viability in the presence of  $\text{TNF-}\alpha$ . The propensity of hypericin to act in association with membranes may imply that TNF type 1 receptor cross-linking (37) is inhibited at the target cell level.

Hypericin is currently being evaluated in clinical trials as an anticancer agent, based on its signal transduction inhibitory properties of cell proliferation. The current findings, which demonstrate interference with cell-mediated cytotoxicity without depleting the organism of effector cells, suggest the possible utilization of hypericin to treat pathological states generated by the cytotoxic effects of T cells. Diseases such as psoriasis, aspects of graft versus host disease, juvenile diabetes and others are potential indications. Clinical symptoms of graft versus host disease, which we induced in mice, were improved by repeated dosing of the animals with hypericin (unpublished data). Hypocrellins, obtained from the fungus *Hypocrella bambuase*, which are natural quinones related to hypericin in structure, physico-chemical and photodynamic properties, are being used in China to treat psoriasis (38,39). This activity of hypocrellins provides an additional indirect incentive to the clinical development of hypericin.

## Acknowledgements

Supported by NIH grant HL 53379-01 and by grant 3181 of the Israeli Health Ministry.

## Abbreviations

BLT	<i>N</i> - $\alpha$ -benzyloxycarbonyl-L-lysine thiobenzyl ester
CTL	cytotoxic T lymphocyte
PHA	phytohemagglutinin
PKC	protein kinase C
PTK	protein tyrosine kinase
TNF	tumor necrosis factor

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