

# Photoactivated Hypericin is an Anti-Proliferative Agent that Induces a High Rate of Apoptotic Death of Normal, Transformed, and Malignant T Lymphocytes: Implications for the Treatment of Cutaneous Lymphoproliferative and Inflammatory Disorders

Floyd E. Fox,<sup>1</sup> Zhutian Niu, Alfonso Tobia,\* and Alain H. Rook

Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.; \*VimRx Pharmaceuticals, Wilmington, Delaware, U.S.A.

Hypericin is a photodynamic compound activated by either visible (400–700 nm) or UVA (320–400 nm) light, and has been shown to inhibit the growth of a variety of neoplastic cell types. In this study, hypericin was found to inhibit proliferative responses of malignant T cells derived from the blood of patients with cutaneous T cell lymphoma. Control cells included peripheral blood mononuclear cells (PBMC) from normal volunteers or Epstein–Barr virus-transformed lymphocytes. Cells from each of these populations were incubated with serial dilutions of hypericin or 8-methoxypsoralen and then stimulated with the mitogen ConA (10 µg per ml). Cultures were prepared in the dark to minimize photoactivation of the hypericin. Proliferation was measured by [<sup>3</sup>H]thymidine labeling after 72 h. Hypericin, photoactivated with 1.1–3.3 J white light per cm<sup>2</sup>, inhibited cellular proliferation of malignant T cells with IC<sub>50</sub> values from 0.34 to 0.53 µM, normal PBMC with IC<sub>50</sub> values of 0.11–0.76 µM, and Epstein–Barr virus-transformed cells with

IC<sub>50</sub> values of 0.75–3.2 µM. UVA-photoactivated hypericin (0.5–2.0 J per cm<sup>2</sup>) could also inhibit proliferation with IC<sub>50</sub> values of 0.57–1.8 µM, 0.7–4.6 µM, and 2.0–3.7 µM for malignant, normal, or Epstein–Barr virus-transformed cells, respectively. Hypericin, photoactivated with either UVA or white light, could induce near complete apoptosis (94%) in malignant cutaneous T cell lymphoma T cells, whereas lower levels of apoptosis (37–88%) were induced in normal PBMC. These data indicate that hypericin inhibits mitogen-induced proliferation of malignant T cells from patients with cutaneous T cell lymphoma, PBMC from normal individuals, as well as Epstein–Barr virus-transformed lymphocytes, and that inhibition of cell proliferation is dependent on the concentration of hypericin used and the dose of light required to photoactivate the compound. Induction of apoptosis is, in part, one mechanism by which photoactivated hypericin inhibits malignant T cell proliferation. *Key words: apoptosis/CTCL/photoactivation. J Invest Dermatol 111:327–332, 1998*

**H**ypericin is a naturally occurring photodynamic compound derived predominantly from the stems, petals, and leaves of the St. John's Wort (*Hypericum perforatum*). It has been demonstrated to possess a number of important biologic properties including potent antiviral activity (Meruelo *et al*, 1988; Lopez-Bazzocchi *et al*, 1991; Carpenter *et al*, 1994). Light activation of hypericin for 15–30 min inactivates infectious titers (5–6 log<sub>10</sub>) of HIV as well as other enveloped and nonenveloped viruses (Meruelo *et al*, 1988; Kraus *et al*, 1990; Tang *et al*, 1990; Carpenter and Kraus, 1991; Hudson *et al*, 1991; Lenard *et al*, 1993). In addition, hypericin inhibits the growth of cells derived from a variety of neoplastic tissues including glioma (Couldwell *et al*,

1994; Zhang *et al*, 1996), neuroblastoma (Zhang *et al*, 1995; Diwu, 1995), adenoma (Hamilton *et al*, 1996), mesothelioma (Koren *et al*, 1996), melanoma (Thomas and Pardini, 1992; Hadjur *et al*, 1996), carcinoma (Andreoni *et al*, 1994; Migita *et al*, 1995; VanderWerf *et al*, 1996), sarcoma (Chung *et al*, 1994; VanderWerf *et al*, 1996), and leukemia (Jarvis *et al*, 1994). Although the mechanism by which hypericin exerts its biologic activity is unclear, it has been demonstrated to inhibit protein kinase activity (Takahashi *et al*, 1989; Zhang *et al*, 1995; Agostinis *et al*, 1996) as well as other enzymes such as monoamine oxidase (Bladt and Wagner, 1994; Perovic and Muller, 1995) and mitochondrial succinoxidase, possibly via generation of singlet oxygen (Thomas *et al*, 1992). Hypericin does not appear to act by intercalating into DNA and thus should not possess some of the deleterious side-effects that have been recently reported with other therapeutic regimens that utilize intercalating photoactive agents such as psoralen (Stern *et al*, 1997).

Cutaneous T cell lymphoma (CTCL) is characterized by the accumulation of clonal malignant CD4<sup>+</sup> T cells in the skin and peripheral blood (Whittaker *et al*, 1991; Zelickson *et al*, 1991; Wolff-Sneedorff *et al*, 1995). An advanced form of CTCL, the Sezary syndrome, presents with erythroderma, lymphadenopathy, and the presence of malignant T cells in the peripheral circulation. The clonal

Manuscript received December 4, 1997; revised March 24, 1998; accepted for publication April 19, 1998.

Reprint requests to: Dr. Alain H. Rook, Department of Dermatology, University of Pennsylvania, 238 CRB/6142–415 Curie Blvd, Philadelphia, PA 19104.

Abbreviations: PBMC, peripheral blood mononuclear cells; TUNEL, terminal dUTP nick end labeling.

<sup>1</sup>Current address: Centocor, 200 Great Valley Pkwy, Malvern, PA 19355.

nature of these malignant T cells has been well established and it appears there is not a restricted use of a particular T cell antigen receptor beta chain (TCR-V $\beta$ ) among patients with Sezary syndrome, although frequently many individuals with Sezary syndrome have an expansion of malignant T cells expressing a unique T cell clonotype (Weiss *et al*, 1989; Lessin *et al*, 1991; Weinberg *et al*, 1995; Dummer *et al*, 1996). It has been suggested that the particular clone of malignant T cells might be responsible for the characteristic immune abnormalities seen in this disease, which include elevated production of Th2 cytokines (IL-4, IL-5, and IL-10), decreased production of Th1 cytokines (IL-2 and IFN- $\gamma$ ), and decreased cell-mediated immunity (Rook *et al*, 1993).

The majority of studies on the biologic activities of hypericin have been performed without specific photoactivation. This study was designed to determine whether chemically synthesized hypericin is capable of inhibiting the proliferative responses to mitogens of normal peripheral blood mononuclear cells (PBMC) or malignant T cells derived from CTCL patients. In addition, we examined whether hypericin could inhibit the proliferation of an Epstein-Barr virus (EBV)-transformed lymphocyte cell line. We also sought to obtain information regarding the concentrations of hypericin necessary to cause inhibition and to determine the qualitative and quantitative nature of light exposure necessary to yield optimal biologic activity of hypericin. Finally, we determined if apoptosis of hypericin-treated cells could explain the observed anti-proliferative activity in the cell systems under study.

#### MATERIALS AND METHODS

**Preparation of cell populations** PBMC were prepared as previously described (Rook *et al*, 1995). Whole blood obtained from normal healthy donors or patients with Sezary Syndrome was diluted 2-fold with Dulbecco's phosphate buffered saline, layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged for 30 min at 500 $\times$ g. The interface containing the mononuclear cell fraction was washed with Dulbecco's phosphate buffered saline by centrifugation and resuspended in complete medium at the indicated cell concentration. In certain experiments, malignant clonal T cells were isolated using TCR clonotypic specific monoclonal antibodies and immunomagnetic beads. Briefly, monocytes were depleted from PBMC by adherence to gelatin-coated tissue culture flasks. The nonadherent fraction was incubated with an anti-TCR V $\beta$  antibody specific for the expanded malignant clone of the particular patient (Immunotech, Westbrook, ME) for 30 min at 4°C. The cells were then washed to remove unbound antibody and then combined with immunomagnetic beads coated with goat anti-mouse Fc specific antibodies (PerSeptive Diagnostics, MA) at a bead:cell ratio of 25:1. Positively selected malignant, clonotypic T cells were recovered in a magnetic field and the selection process repeated on the cells not binding to the beads in this first round of separation. The TCR V $\beta$ -positively selected cells were pooled and were determined to be greater than 97% pure by flow cytometric analysis. The remaining T cells, comprising the unselected clonotypes, were utilized as a control for the malignant cells in appropriate experiments.

**Reagents** Chemically synthesized hypericin was supplied by VIMRX Pharmaceuticals (Wilmington, DE). A stock solution was prepared by dissolving 40 mg Hypericin in 40 ml 2% benzyl alcohol (vehicle) to yield a 2 mM solution and kept in the dark at 4°C. Eight-methoxypsoralen (8-MOP) was purchased from Sigma (St. Louis, MO) and a stock solution of 462.5  $\mu$ M was prepared by dissolving 100  $\mu$ g 8-MOP in 1 ml 2% benzyl alcohol and keeping in the dark at 4°C.

**Cell culture and photoactivation** Purified cells from normal donors or patients were diluted to a concentration of  $2 \times 10^6$  cells per ml with complete medium and 100  $\mu$ l were aliquoted into each well of a 96 well microtiter plate. Medium, vehicle, hypericin, or 8-MOP were 5-fold serially diluted into their respective set of wells in a darkened tissue culture room (subdued light). One plate that was to receive no exposure to photoactivating wavelengths of light was placed immediately in a 37°C incubator. The remaining plates were exposed to white light from four fluorescent F15T8CW 15 W bulbs 9 cm above the tissue culture plates. Photoactivation with fluorescent illumination lasted for between 10 and 30 min and delivered the equivalent of 1.1–3.3 J per cm $^2$ . Alternatively, photoactivation was performed with UVA illumination (320–400 nm) on a light box utilizing eight UVA-AR bulbs. Photoactivation with UVA light lasted between 0.5 and 4 min and delivered the equivalent of 0.5–2.0 J per cm $^2$ . The plates were then incubated at 37°C for 24 h or 72 h depending on the assay to be performed. To quantitate the light delivered to the medium containing well of a tissue culture plate an IL-700 A research radiometer (International Light, Newburyport, MA) was used.

**Proliferation assay** Proliferation was measured by quantitating the incorporation of tritiated thymidine ( $^3$ H]TdR) into newly synthesized DNA. Briefly, control or treated cells were cultured at  $2 \times 10^6$  cells per ml in the presence of ConA (10  $\mu$ g per ml) for 72 h at 37°C in a humidified, 5% CO $_2$  incubator in complete medium. During the final 18 h of culture, 0.5 uCi of  $^3$ H]TdR was added to each well. The cells were harvested onto glass fiber filters using a Skatron Cell Harvester. Incorporated cpm was then quantitated on a scintillation counter and plotted as shown in the figures.

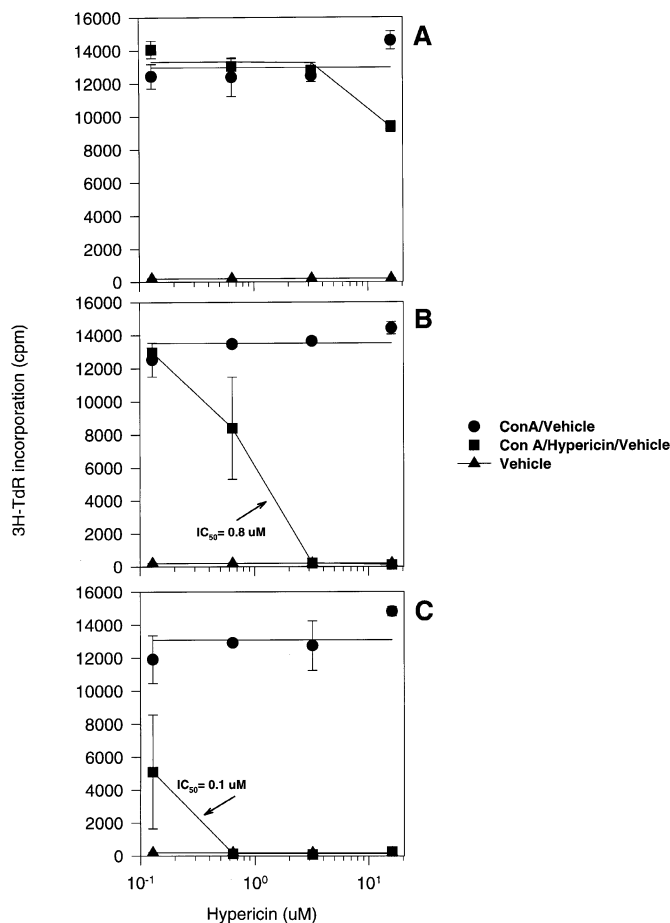
**Apoptosis assay** Apoptosis was assessed using a modification of the terminal dUTP nick end labeling (TUNEL) method previously described by Sgonc *et al* (1994), as supplied in kit form by Boehringer (Indianapolis, IN). Apoptotic cell death is characterized by the internucleosomal cleavage of DNA into fragments of  $\approx$ 180 bp. The TUNEL assay allows for the incorporation of fluorescein labeled-dUTP (FITC-dUTP) into the free ends of these apoptotic DNA fragments. In addition, by using a flow cytometric analysis, apoptotic cells can be distinguished from necrotic cells because apoptotic cells will retain their cell membrane integrity (and relative size) that is reflected in little or no change in their forward light scattering properties. Cells that do not change in size and incorporate FITC-dUTP label can reliably be identified as apoptotic. Briefly, cells cultured as described above were fixed in 4% paraformaldehyde for 30 min at 25°C, and permeabilized in 0.1% Triton containing 0.1% sodium citrate for 2 min on ice. Terminal deoxynucleotide transferase (vial 1) and nucleotide mixture (vial 2) were then added to the cells and the mixture was incubated for 60 min at 37°C. Then, using a FACScan flow cytometer (Becton Dickinson), the percentage of cells (of the 10,000 total acquired) undergoing apoptosis was determined as:

$$\frac{\text{Number of cells incorporating FITC-dUTP into fragmented DNA}}{\text{Total number of cells}} \times 100$$

#### RESULTS

**Photoactivation of hypericin with white light potentiates its anti-proliferative effect** To examine the role of photoactivated hypericin on proliferative responses of lymphocytes to mitogenic stimulation, normal, neoplastic, or EBV-transformed lymphocytes were cultured with or without increasing amounts of hypericin. These cultures were kept in subdued light to minimize any photoactivation of hypericin or were illuminated with white light for increasing amounts of time. When normal lymphocytes were kept in subdued light (**Fig 1A**), hypericin induced a small but significant inhibition of proliferation of these cells. We observed that benzyl alcohol vehicle toxicities at higher concentrations began interfering with the hypericin mediated inhibition of lymphocyte proliferation, making an exact calculation of an IC $_{50}$  for nonphotoactivated hypericin impossible. An approximation of the minimum calculated IC $_{50}$  for nonphotoactivated hypericin would be of the order of 22  $\mu$ M. When similar cultures were illuminated with white light for 10 min (1.1 J per cm $^2$ ), the IC $_{50}$  for hypericin was 0.8  $\mu$ M ( $\approx$ 27-fold increase) (**Fig 1B**). Complete inhibition of proliferation of normal lymphocytes was achieved by  $\approx$ 3.2  $\mu$ M hypericin and 1.1 J light per cm $^2$ . Increasing the light exposure (duration of photoactivation) with white light to 20 min (2.2 J per cm $^2$ ) (**Fig 1C**) decreased the IC $_{50}$  for hypericin to 0.1  $\mu$ M ( $\approx$ 220-fold increase), and complete inhibition of cell proliferation at this light dose was observed at a concentration of 0.64  $\mu$ M hypericin. The increased level of inhibition observed when hypericin-treated lymphocytes were illuminated for 20 min, compared with illumination for 10 min, suggests that there is an optimal activation time needed for maximal efficacy of hypericin. Illumination of hypericin-treated lymphocytes for 30 min (IC $_{50}$  = 0.17  $\mu$ M, data not shown) did not significantly increase the inhibition of cell proliferation, which suggests that the optimal anti-proliferative activity can be elicited when hypericin is photoactivated for between 10 and 20 min (1.1–2.2 J per cm $^2$ ).

Hypericin photoactivation studies utilizing peripheral blood cells from a patient having leukemic CTCL (Sezary syndrome) are depicted in **Fig 2**. Previous characterization of this patient's peripheral blood lymphocytes by flow cytometry indicated that 99% of these cells were CD4 $^+$ /CD8 $^-$ /V $\beta$ 22 $^+$ , suggesting that the vast majority had an abnormal phenotype that was contained within a single clonal population. In cultures of these lymphocytes, which were illuminated for 20 min, the IC $_{50}$  for hypericin was 0.3  $\mu$ M, and complete inhibition of cell proliferation was observed at a concentration of 3.2  $\mu$ M hypericin

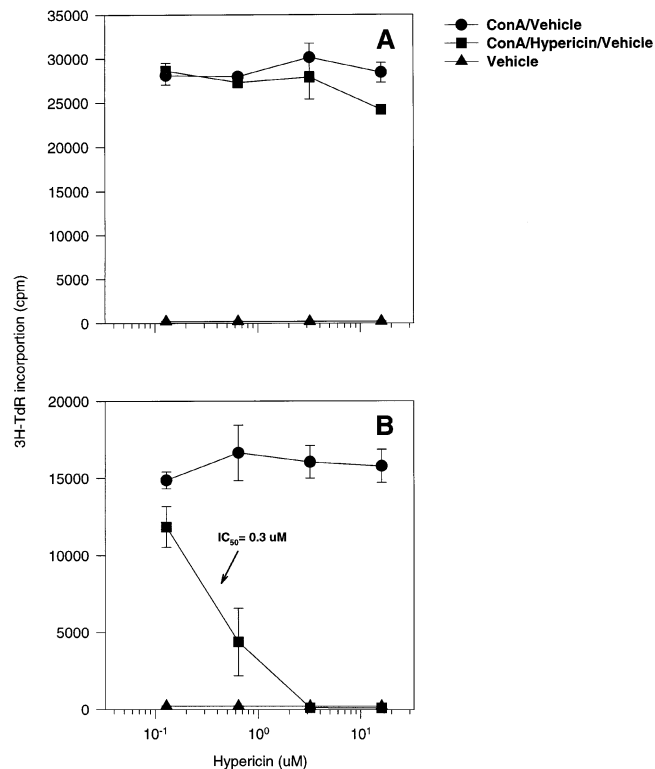


**Figure 1. Inhibition of normal PBMC proliferation by photoactivating hypericin with white light.** PBMC from normal individuals were incubated with ConA (10  $\mu\text{g}$  per ml) with or without 5-fold serial dilutions of hypericin and at the indicated concentrations. Cultures were then left (A) unexposed, (B) exposed to 10 min (1.1 J per  $\text{cm}^2$ ) white light, or (C) exposed to 20 min (2.2 J per  $\text{cm}^2$ ) white light prior to culturing for a further 72 h. Proliferation as determined by the amount of newly synthesized DNA present was quantitated during the final 18 h of culture by measuring incorporation of [ $^3\text{H}$ ]TdR. Data are represented as the mean  $\pm$  SD of triplicate points from the same individual.

(Fig 2B). In cultures of Sezary lymphocytes that were kept in subdued light, an approximation of the minimum  $\text{IC}_{50}$  for nonphotoactivated hypericin would be 34  $\mu\text{M}$  (Fig 2A). These data indicate that hypericin inhibits mitogen-induced proliferation of normal and malignant human PBMC.

We also examined the ability of photoactivated hypericin to inhibit the proliferation of cells transformed with EBV because this represents a model of a B cell lymphoproliferative disorder (Fig 3B). In cultures of EBV-transformed lymphocytes illuminated for 10 min with white light, the  $\text{IC}_{50}$  for hypericin was 3.2  $\mu\text{M}$  (11.3-fold over nonphotoactivated). In cultures of transformed lymphocytes illuminated for 30 min, the  $\text{IC}_{50}$  for hypericin was 0.75  $\mu\text{M}$  (48-fold over nonphotoactivated), and complete inhibition of cell proliferation was observed at a concentration of 3.2  $\mu\text{M}$  hypericin. In contrast, in cultures of transformed lymphocytes that were not illuminated, the approximate minimum  $\text{IC}_{50}$  for nonphotoactivated hypericin would be 36  $\mu\text{M}$ . In contrast to the results obtained using normal lymphocytes, illumination of transformed lymphocytes for a period of 30 min resulted in inhibition of proliferation greater than that observed from photoactivation for periods of 10 or 20 min (Fig 3B). These results indicate that the optimal period of photoactivation of hypericin may be expected to vary, depending upon the characteristics of the lymphocytes or cells to be affected.

**Photoactivation of hypericin with ultraviolet light also potentiates its anti-proliferative effect** Because hypericin exhibits absorp-

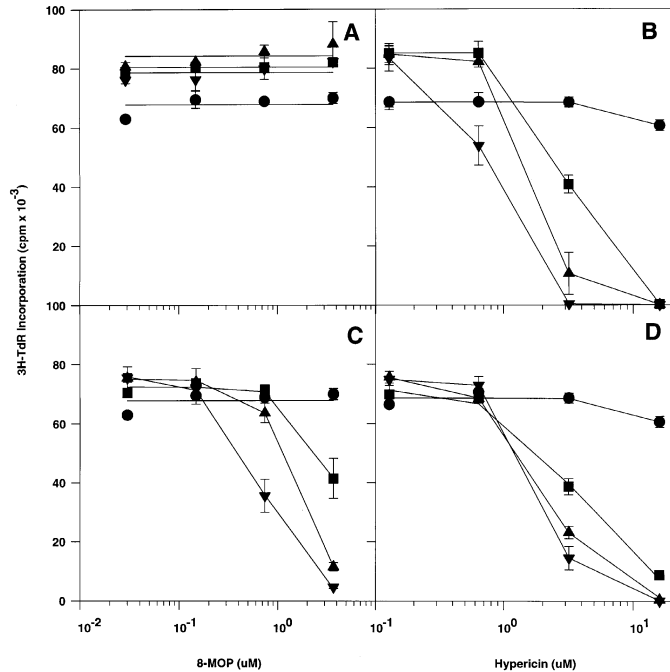


**Figure 2. Inhibition of malignant T cell proliferation by photoactivating hypericin with white light.** Malignant T cells from patients with CTCL were incubated with ConA (10  $\mu\text{g}$  per ml) with or without 5-fold serial dilutions of hypericin at the indicated concentrations. Cultures were then left (A) unexposed or (B) exposed to 20 min white light prior to culturing for a further 72 h. Proliferation as determined by the amount of newly synthesized DNA present was quantitated during the final 18 h of culture by measuring incorporation of [ $^3\text{H}$ ]TdR. Data are represented as the mean  $\pm$  SD of triplicate points from the same individual.

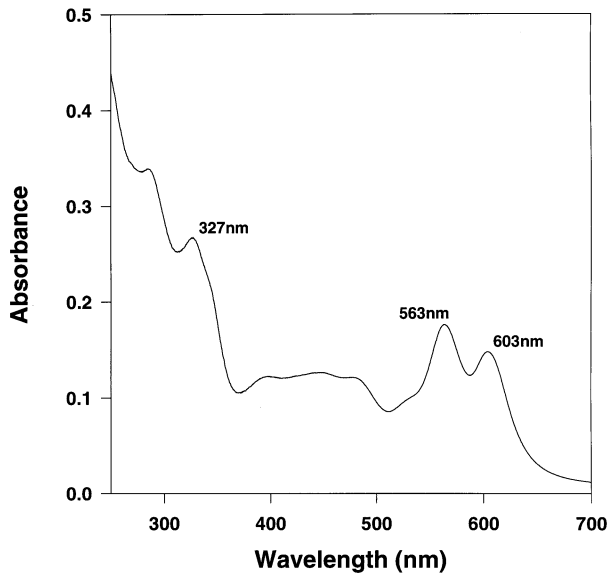
ence peaks in the UVA region (327 nm) of the electromagnetic spectrum as well as the visible region (563 and 603 nm) (Fig 4), we wanted to determine the ability of hypericin to inhibit proliferation of lymphocytes upon photoactivation with UVA light. In addition, we compared the anti-proliferative effects of 8-MOP, another UVA photoactivatable compound, to those of hypericin.

The ability of 8-MOP to inhibit proliferation of EBV-transformed lymphocytes was not evident and thus was not enhanced upon illumination using white light (Fig 3A). In contrast, as indicated by the data depicted in Fig 3(B), the ability of hypericin to inhibit proliferation of EBV-transformed lymphocytes was enhanced significantly upon photoactivation with white light. In the absence of white light illumination, the approximate minimum  $\text{IC}_{50}$  for hypericin was calculated to be 36  $\mu\text{M}$ ; following 30 min of white light illumination, the  $\text{IC}_{50}$  for hypericin was 0.75  $\mu\text{M}$ .

The ability of both hypericin and 8-MOP to inhibit proliferation of EBV-transformed lymphocytes was enhanced significantly upon illumination using UVA light. In the absence of UVA illumination, the approximate minimum  $\text{IC}_{50}$  for 8-MOP could not be calculated and the approximate minimum  $\text{IC}_{50}$  for hypericin (Fig 3D) was calculated to be 36  $\mu\text{M}$ ; following 2 J UVA illumination per  $\text{cm}^2$ , the  $\text{IC}_{50}$  for 8-MOP (Fig 3C) was 0.7  $\mu\text{M}$  and the  $\text{IC}_{50}$  for hypericin was 2.0  $\mu\text{M}$ . Furthermore, the ability of hypericin to inhibit cell proliferation was essentially the same whether 2 or 1 J UVA illumination per  $\text{cm}^2$  was applied. In contrast, the ability of 8-MOP to inhibit cell proliferation roughly doubled when 2 J UVA illumination per  $\text{cm}^2$  was applied, relative to when 1 J UVA illumination per  $\text{cm}^2$  was applied (at the 0.7  $\mu\text{M}$  concentration). These results indicate that less UVA illumination is required to elicit the maximal anti-proliferative effect of hypericin than is necessary to elicit the maximal anti-proliferative activity of 8-MOP.

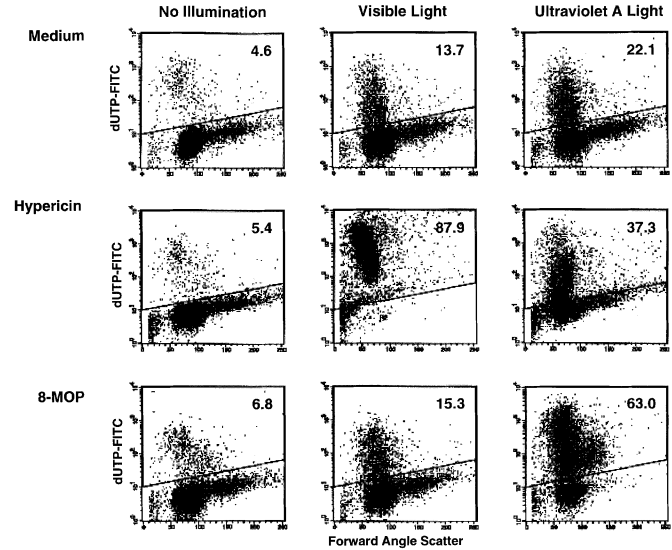


**Figure 3. Effect of photoactivation with visible or UVA light on hypericin or 8-MOP inhibition of EBV-transformed B lymphocyte proliferation.** EBV-transformed lymphocytes were incubated in complete medium with or without 5-fold serial dilutions of hypericin (B, D) or 8-MOP (A, C) at the indicated concentrations. (A, B) Cultures were then left unexposed (l) or exposed to 10 min [1.1 J per cm<sup>2</sup>] (n), 20 min [2.2 J per cm<sup>2</sup>] (s), or 30 min [3.3 J per cm<sup>2</sup>] (t) of white light prior to culturing for a further 72 h. (C, D) Cultures were then left unexposed (l) or exposed to 0.5 J per cm<sup>2</sup> (n), 1.0 J per cm<sup>2</sup> (s), or 2.0 J per cm<sup>2</sup> (t) of UVA light prior to culturing for a further 72 h. Proliferation as determined by the amount of newly synthesized DNA present was quantitated during the final 18 h of culture by measuring incorporation of [<sup>3</sup>H]TdR. Data are represented as the mean ± SD of triplicate points from the same individual.

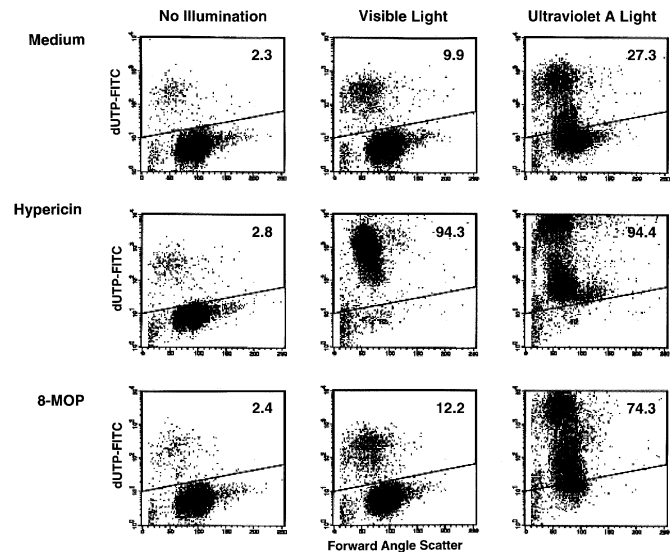


**Figure 4. Absorbance spectrum of 20  $\mu$ M hypericin in phosphate buffered saline.** Hypericin stock solution was dissolved in phosphate buffered saline to a final concentration of 20  $\mu$ M and a spectrum was acquired using a Beckman DU-640 spectrophotometer.

**Induction of apoptosis of lymphocytes using photoactivated hypericin** In an effort to investigate the mechanisms of the anti-proliferative effects of hypericin, we determined whether hypericin treated lymphocytes were induced to undergo apoptosis. The ability



**Figure 5. Induction of apoptosis in normal PBMC by photoactivated hypericin or 8-MOP.** Medium (row 1), 10  $\mu$ M hypericin (row 2), or 0.462  $\mu$ M (100 ng per ml) 8-MOP (row 3) were added to cultures of PBMC from normal individuals. These cultures were then left unexposed (column 1) or exposed to 20 min white light (column 2) or 2 J UVA light per cm<sup>2</sup> (column 3). Cultures were then placed at 37°C for 24 h. Percentage apoptosis was determined using a TUNEL assay as described in *Materials and Methods*.



**Figure 6. Induction of apoptosis in malignant T cells by photoactivated hypericin or 8-MOP.** Medium (row 1), 10  $\mu$ M hypericin (row 2), or 0.462  $\mu$ M (100 ng per ml) 8-MOP (row 3) were added to cultures of malignant T cells from individuals with CTCL. These cultures were then left unexposed (column 1) or exposed to 20 min white light (column 2) or 2 J UVA light per cm<sup>2</sup> (column 3). Cultures were then placed at 37°C for 24 h. Percentage apoptosis was determined using a TUNEL assay as described in *Materials and Methods*.

of photoactivated hypericin to induce apoptosis in PBMC from a representative normal individual (Fig 5) or neoplastic lymphocytes from a patient with CTCL (Fig 6) was compared with the ability of photoactivated 8-MOP to induce apoptosis in these cells. Hypericin-treated, 8-MOP-treated, or untreated normal lymphocytes or malignant T cells were photoactivated using white light for 20 min or 2 J UVA light per cm<sup>2</sup>, or were not photoactivated. After 18 h, apoptotic cells (cells above the diagonal line) were distinguished from nonapoptotic cells based on increased dUTP-FITC incorporation using flow cytometry. Normal or neoplastic cells incubated with either hypericin or 8-MOP did not induce a significant increase in apoptosis in the absence of photoactivation (Figs 5, 6, column 1). Hypericin photoactivated

**Table I. Induction of apoptosis in V $\beta$ 8<sup>+</sup> and V $\beta$ 8<sup>-</sup> CTCL T cells by photoactivated hypericin or 8-MOP<sup>a</sup>**

	Photoactivation source					
	None		2.2 J per cm <sup>2</sup> visible		2 J per cm <sup>2</sup> UVA	
	V $\beta$ 8 <sup>+</sup>	V $\beta$ 8 <sup>-</sup>	V $\beta$ 8 <sup>+</sup>	V $\beta$ 8 <sup>-</sup>	V $\beta$ 8 <sup>+</sup>	V $\beta$ 8 <sup>-</sup>
Vehicle	18	7	18	20	35	ND
Hypericin, 10 $\mu$ M	21	8	96	90	96	80
8-MOP, 10 $\mu$ M	16	5	16	18	85	83

<sup>a</sup>The malignant clonotypic T cells (TCR V $\beta$ 8<sup>+</sup> cells) were positively selected from the nonmalignant T cells (TCR V $\beta$ 8<sup>-</sup> cells) in an individual with a previously identified clonal T cell population as described in *Materials and Methods*. The two populations were incubated with vehicle or either hypericin (10  $\mu$ M) or 8-MOP (10  $\mu$ M) in the dark (no exposure) or followed by photoactivation with either visible or UVA light. Apoptosis was measured 24 h later using the TUNEL procedure. Values represent the percentage of V $\beta$ 8<sup>+</sup> or V $\beta$ 8<sup>-</sup> cells undergoing apoptosis. ND, not done.

with white light induced apoptosis in nearly all normal and malignant cells (87.9 and 94.3%, respectively, **Figs 5, 6**). The completeness of apoptosis induction can also be seen by a disappearance of the cells below the diagonal. This occurs only in cultures treated with hypericin and subsequent photoactivation. White light-activated 8-MOP induced apoptosis in a minimal number of each cell type (15.3% and 12.2%, respectively). Malignant cells treated with hypericin or 8-MOP photoactivated with 2 J UVA per cm<sup>2</sup> (**Fig 6, column 3**) demonstrated induction of apoptosis levels near or comparable with hypericin photoactivated with white light. Normal cells treated with hypericin or 8-MOP photoactivated with 2 J UVA per cm<sup>2</sup> (**Fig 5, column 3**) demonstrated significant induction of apoptosis but at levels lower than that of hypericin photoactivated with white light.

To investigate whether hypericin was inducing apoptosis preferentially in malignant versus nonmalignant T cells present in the blood of individuals with CTCL, we positively selected the malignant clonotypic T cells (TCR V $\beta$ 8<sup>+</sup> cells) from the nonmalignant T cells (TCR V $\beta$ 8<sup>-</sup> cells) from an individual with a previously identified clonal T cell population. These two populations were incubated with vehicle or either hypericin (10  $\mu$ M) or 8-MOP (10  $\mu$ M) in the dark (no exposure) or followed by photoactivation with either visible or UVA light. As can be seen in Table I, in cultures kept in the dark there is some increased level of apoptosis in V $\beta$ 8<sup>+</sup> cells when compared with V $\beta$ 8<sup>-</sup> cells (16–21% vs 5–8%). In cultures photoactivated with white light, hypericin does not appear to selectively induce apoptosis in the malignant versus nonmalignant T cells; however, in the presence of UVA, hypericin induces greater levels of apoptosis in V $\beta$ 8<sup>+</sup> cells than in V $\beta$ 8<sup>-</sup> cells (96% vs 80%), whereas 8-MOP does not appear to have any selective effect. In addition, these data also suggest that hypericin photoactivated with either visible or UVA light may be a more potent inducer of malignant T cell apoptosis than 8-MOP.

## DISCUSSION

Hypericin is a photodynamic agent that has documented activity against a wide variety of neoplastic cells (Thomas and Pardini, 1992; Couldwell *et al*, 1994; Andreoni *et al*, 1994; Chung *et al*, 1994; Jarvis *et al*, 1994; Zhang *et al*, 1995, 1996; Diwu, 1995; Migita *et al*, 1995; Hamilton *et al*, 1996; Koren *et al*, 1996; Hadjur *et al*, 1996; VanderWurf *et al*, 1996). In the presence of light, hypericin has been described to excite oxygen to its singlet state (Duran and Song, 1986; Thomas *et al*, 1992; Diwu and Lown, 1993). In addition, hypericin has been shown to be involved in the generation of superoxide anion radicals (Diwu and Lown, 1993). Hypericin can be optimally photoactivated by light of about 570–650 nm wavelength, i.e., in the yellow region (Meruelo *et al*, 1988; Lavie *et al*, 1989; Thomas *et al*, 1992), but can also be activated by UVA light (**Figs 3, 5, 6**) to potentiate its biologic activity. Hypericin binds to phospholipids such as phosphatidylcholine of cell membranes, and it binds to retroviral particles, probably by associating with the membrane-derived lipid envelope (Lavie *et al*, 1995).

Recent data also suggest that hypericin is an inhibitor of protein tyrosine and serine/threonine kinase activity (deWitte *et al*, 1993; Agostinis *et al*, 1996). Specifically, hypericin has been demonstrated to inhibit the action of protein kinase C (Takahashi *et al*, 1989; Zhang *et al*, 1995; Utsumi *et al*, 1995; Hamilton *et al*, 1996) through direct interaction with the regulatory domain of the enzyme, although there is at least one report of it having no inhibitory effect on PKC activity (Jarvis *et al*, 1994). Hypericin was not photoactivated, however, which may account for its lack of PKC inhibitory activity in that study. Direct interaction of hypericin with PKC might be one potential mechanism by which hypericin can induce apoptosis of lymphoid cells. Several compounds (i.e., H7, staurosporine) that can inhibit PKC activity have been shown to cause various neoplastic cell types to undergo apoptosis (Jarvis *et al*, 1994; Zhang *et al*, 1995). It is also possible that photoactivated hypericin may be acting indirectly, through the generation of singlet oxygen, to inhibit proliferation and induce apoptosis. Whether singlet oxygen generation resulting from photoactivation of hypericin is causally related to inhibition of PKC and induction of apoptosis is not known at this time. Nevertheless, at this time it is not clear what role singlet oxygen may have on protein kinase activity.

A possible mechanism by which the leukemic T cells in CTCL accumulate in the skin and blood may be due to deficient or absent apoptosis, because they do not exhibit strong spontaneous proliferation as measured by [<sup>3</sup>H]TdR incorporation. Implicit in the results of this study are that the topical or systemic administration of hypericin, followed by photoactivation, could lead to a beneficial therapeutic effect in CTCL by eliminating malignant T cells through the induction of apoptosis. Moreover, other inflammatory skin diseases typified by lymphocytic infiltrates, including psoriasis and lichen planus, might also be treated with topical hypericin.

Hypericin has several attributes that make it particularly attractive for investigating its clinical use in skin disorders with potential advantages over phototherapy with psoralen plus UVA. Hypericin has been shown to possess little toxicity when administered systemically (Meruelo *et al*, 1988), and is maximally activated by white light at wavelengths produced by the sodium lamp (590 nm), which significantly reduces the side-effects associated with UV irradiation. There have been several recent reports that UVA exposure, particularly in the course of psoralen plus UVA therapy, may have long-term detrimental effects including mutagenesis and skin cancer development (Stern *et al*, 1997). Whether these detrimental effects occur as a result of the primary irradiation or as a result of secondary immunosuppression is still under investigation (Wang *et al*, 1997; Van de Kerkhof and de Rooij, 1997). Hence, the use of hypericin potentially permits minimization of exposure to UV illumination and therefore minimization of the undesirable effects of UVA illumination. In addition, hypericin has a relatively long half-life (20–24 h) that allows for repeated light activation with single doses (Liebes *et al*, 1991; Staffeldt *et al*, 1994). Hypericin has been demonstrated to be present in skin after systemic administration and it has been shown to be about 20% bioavailable after oral administration (Kerb *et al*, 1996). Furthermore, the mechanism of photoreactions (singlet oxygen production and apoptosis) are the same as other phototherapeutic agents that have been shown to be beneficial in the treatment of inflammatory skin diseases.

A phase I safety study with normal volunteers using photoactivated hypericin has been completed (data on file, VimRx Pharmaceuticals, Wilmington, DE) and a clinical trial using hypericin as photodynamic therapy to treat inflammatory skin diseases has been initiated. The potential clinical relevance of this interesting agent will soon be understood.

## REFERENCES

- Agostinis P, Donella-Deana A, Cuveele J, Vandenbogaerde A, Samo S, de Merlevede W: A comparative analysis of the photosensitized inhibition of growth-factor regulated protein kinases by hypericin-derivatives. *Biochem Biophys Res Commun* 220:613–617, 1996
- Andreoni A, Colasanti A, Colasanti P, Mastrocinque M, Riccio P, Roberti G: Laser photosensitization of cells by hypericin. *Photochem Photobiol* 59:529–533, 1994
- Bladt S, Wagner H: Inhibition of MAO by fractions and constituents of hypericum extract. *J Geriatric Psychiatry Neurol* 7 (Suppl. 1):S57–S59, 1994

- Carpenter S, Kraus GA: Photosensitization is required for inactivation of equine infectious anemia virus by hypericin. *Photochem Photobiol* 53:169-174, 1991
- Carpenter S, Fehr MJ, Kraus GA, Petrich JW: Chemiluminescent activation of the antiviral activity of hypericin: a molecular flashlight. *Proc Natl Acad Sci USA* 91:12273-12277, 1994
- Chung PS, Saxton RE, Paiva MB, et al: Hypericin uptake in rabbits and nude mice transplanted with human squamous cell carcinomas: study of a new sensitizer for laser phototherapy. *Laryngoscope* 104:1471-1476, 1994
- Couldwell WT, Gopalakrishna R, Hinton DR, He S, Weiss MH, Law RE, Apuzzo ML: Hypericin: a potential antiangioma therapy [published erratum appears in *Neurosurgery* 35:993, 1994]. *Neurosurgery* 35:705-710, 1994
- Diwu Z: Novel therapeutic and diagnostic applications of hypocrellins and hypericins. *Photochem Photobiol* 61:529-539, 1995
- Diwu Z, Lown JW: Photosensitization with anticancer agents 17: The EPR study of photodynamic action of hypericin. *Free Rad Biol Med* 14:209-215, 1993
- Dummer R, Heald PW, Nestle FO, Ludwig E, Laine E, Hemmi S, Burg G: Sezary syndrome T cell clones display T-helper 2 cytokines and express the accessory factor-1 (interferon-gamma receptor beta-chain). *Blood* 88:1383-1389, 1996
- Duran N, Song PS: Hypericin and its photodynamic action. *Photochem Photobiol* 43:677-680, 1986
- Hadjir C, Richard MJ, Parat MO, Jardon P, Favier A: Photodynamic effects of hypericin on lipid peroxidation and antioxidant status in melanoma cells. *Photochem Photobiol* 64:375-381, 1996
- Hamilton HB, Hinton DR, Law RE, et al: Inhibition of cellular growth and induction of apoptosis in pituitary adenoma cell lines by the protein kinase C inhibitor hypericin: potential therapeutic application. *J Neurosurgery* 85:329-334, 1996
- Hudson JB, Lopez-Bazzocchi I, Towers GH: Antiviral activities of hypericin. *Antiviral Res* 15:101-112, 1991
- Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S: Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. *Cancer Res* 54:1707-1714, 1994
- Kerb R, Brockmoller J, Staffeldt B, Ploch M, Roots I: Single-dose and steady-state pharmacokinetics of hypericin and pseudohypericin. *Antimicrob Agents Chemother* 40:2087-2093, 1996
- Koren H, Schenk GM, Jindra RH, et al: Hypericin in phototherapy. *J Photochem Photobiol B - Biol* 36:113-119, 1996
- Kraus GA, Pratt D, Tossberg J, Carpenter S: Antiretroviral activity of synthetic hypericin and related analogs. *Biochem Biophys Res Commun* 172:149-153, 1990
- Lavie G, Valentine F, Levin B, et al: Studies of the mechanisms of action of the antiretroviral agents hypericin and pseudohypericin. *Proc Natl Acad Sci USA* 86:5963-5967, 1989
- Lavie G, Mazur Y, Lavie D, Meruelo D: The chemical and biological properties of hypericin - a compound with a broad spectrum of biological activities [published erratum appears in *Med Res Rev* 15:259, 1995]. *Med Res Rev* 15:111-119, 1995
- Lenard J, Rabson A, Vanderoef R: Photodynamic inactivation of infectivity of human immunodeficiency virus and other enveloped viruses using hypericin and rose bengal: inhibition of fusion and syncytia formation. *Proc Natl Acad Sci USA* 90:158-162, 1993
- Lessin SR, Rook AH, Rovera G: Molecular diagnosis of cutaneous T cell lymphoma: polymerase chain reaction amplification of T cell antigen receptor beta-chain gene rearrangements. *J Invest Dermatol* 96:299-302, 1991
- Liebes L, Mazur Y, Freeman D, et al: A method for the quantitation of hypericin, an antiviral agent, in biological fluids by high-performance liquid chromatography. *Anal Biochem* 195:77-85, 1991
- Lopez-Bazzocchi I, Hudson JB, Towers GH: Antiviral activity of the photoactive plant pigment hypericin. *Photochem Photobiol* 54:95-98, 1991
- Meruelo D, Lavie G, Lavie D: Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. *Proc Natl Acad Sci USA* 85:5230-5234, 1988
- Migita K, Eguchi K, Kawabe Y, Tsukada T, Ichinose Y, Nagataki S: Defective TCR-mediated signaling in anergic T cells. *J Immunol* 155:5083-5087, 1995
- Perovic S, Muller WE: Pharmacological profile of hypericum extract. Effect on serotonin uptake by postsynaptic receptors. *Arzneimittel-Forschung* 45:1145-1148, 1995
- Rook AH, Lessin SR, Jaworsky C, Singh A, Vowels BR: Immunopathogenesis of cutaneous T cell lymphoma: Abnormal cytokine production by Sezary T cells. *Arch Dermatol* 129:486-489, 1993
- Rook AH, Kubin M, Cassin M, et al: IL-12 reverses cytokine and immune abnormalities in Sezary syndrome. *J Immunol* 154:1491-1498, 1995
- Sgonc R, Boeck G, Dietrich H, Gruber J, Recheis H, Wick G: Simultaneous determination of cell surface antigens and apoptosis. *Trends Genet* 10:41-42, 1994
- Staffeldt B, Kerb R, Brockmoller J, Ploch M, Roots I: Pharmacokinetics of hypericin and pseudohypericin after oral intake of the hypericum perforatum extract LI 160 in healthy volunteers. *J Geriatric Psychiatry Neurol* 7 (Suppl. 1):S47-S53, 1994
- Stern RS, Nichols KT, Vakeva LH: Malignant melanoma in patients treated for psoriasis with methoxsalen (psoralen) and ultraviolet A radiation (PUVA). *New England J Med* 336:1041-1045, 1997
- Takahashi I, Nakanishi S, Kobayashi E, Nakano H, Suzuki K, Tamaoki T: Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antiretroviral activity. *Biochem Biophys Res Commun* 165:1207-1212, 1989
- Tang J, Colacino JM, Larsen SH, Spitzer W: Virucidal activity of hypericin against enveloped and non-enveloped DNA and RNA viruses. *Antiviral Res* 13:313-325, 1990
- Thomas C, Pardini RS: Oxygen dependence of hypericin-induced phototoxicity to EMT6 mouse mammary carcinoma cells. *Photochem Photobiol* 55:831-837, 1992
- Thomas C, MacGill RS, Miller GC, Pardini RS: Photoactivation of hypericin generates singlet oxygen in mitochondria and inhibits succinoxidase. *Photochem Photobiol* 55:47-53, 1992
- Utsumi T, Okuma M, Kanno T, Yasuda T, Kobuchi H, Horton AA, Utsumi K: Light-dependent inhibition of protein kinase C and superoxide generation of neutrophils by hypericin, an antiretroviral agent. *Arch Biochem Biophys* 316:493-497, 1995
- Van de Kerkhof PC, de Rooij MJ: Multiple squamous cell carcinomas in a psoriatic patient following high-dose photochemotherapy and cyclosporin treatment: response to long-term acitretin maintenance. *Br J Dermatol* 136:275-278, 1997
- VanderWerf QM, Saxton RE, Chang A, et al: Hypericin: a new laser phototargeting agent for human cancer cells. *Laryngoscope* 106:479-483, 1996
- Wang XM, McNiff JM, Klump V, Asgari M, Gasparro FP: An unexpected spectrum of p53 mutations from squamous cell carcinomas in psoriasis patients treated with PUVA. *Photochem Photobiol* 66:294-299, 1997
- Weinberg JM, Jaworsky C, Benoit BM, Telegan B, Rook AH, Lessin SR: The clonal nature of circulating Sezary cells. *Blood* 86:4257-4262, 1995
- Weiss LM, Wood GS, Hu E, Abel EA, Hoppe RT, Sklar J: Detection of clonal T cell receptor gene rearrangements in the peripheral blood of patients with mycosis fungoides/Sezary syndrome. *J Invest Dermatol* 92:601-604, 1989
- Whittaker SJ, Smith NP, Jones RR, Luzzatto L: Analysis of beta, gamma, and delta T cell receptor genes in mycosis fungoides and Sezary syndrome. *Cancer* 68:1572-1582, 1991
- deWitte P, Van Agostinis P, LJ, Merlevede W, Vandenheede JR: Inhibition of epidermal growth factor receptor tyrosine kinase activity by hypericin. *Biochemical Pharmacology* 46:1929-1936, 1993
- Wolff-Sneedorff A, Ralfkiaer E, Thomsen K, Vejlsgaard GL: Analyses of T cell receptor beta-chain genes by Southern blotting in known and suspected cutaneous T cell lymphoma. A study of 67 samples from 32 patients. *Clin Exp Dermatol* 20:115-122, 1995
- Zelickson BD, Peters MS, Muller SA, Thibodeau SN, Lust JA, Quam LM, Pittelkow MR: T cell receptor gene rearrangement analysis: cutaneous T cell lymphoma, peripheral T cell lymphoma, and premalignant and benign cutaneous lymphoproliferative disorders. *J Am Acad Dermatol* 25:787-796, 1991
- Zhang W, Lawa RE, Hinton DR, Su Y, Couldwell WT: Growth inhibition and apoptosis in human neuroblastoma SK-N-SH cells induced by hypericin, a potent inhibitor of protein kinase C. *Cancer Lett* 96:31-35, 1995
- Zhang W, Hinton DR, Surnock AA, Couldwell WT: Malignant glioma sensitivity to radiotherapy, high-dose tamoxifen, and hypericin: corroborating clinical response in vitro: case report. *Neurosurgery* 38:587-591, 1996