

ANTIMETASTATIC ACTIVITY OF THE PHOTODYNAMIC AGENT HYPERICIN IN THE DARK

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A unique property of the photodynamic signal transduction inhibitor hypericin (HY) is high functionality in the dark, which has been shown to result in portfolio of anticancer activities both *in vitro* and *in vivo*. Here we show that treatment with HY significantly reduces growth rate of metastases in 2 murine models: breast adenocarcinoma (DA3) and squamous cell carcinoma (SQ2). Focus on metastases was achieved by resection of primary tumors at stages in which micrometastases exist in lungs. Long-term animal survival in DA3 tumor-excised groups increased from 15.6% in controls to 34.5% following supplementary treatment with HY. In mice bearing SQ2 tumor metastases, therapy with HY increased animal survival from 17.7% in controls to 46.1%. Using Laser-induced fluorescence and multipixel spectral image analyses, we demonstrate that HY has a high tendency to accumulate in primary and metastatic tumors; HY content in lungs bearing metastases was approximately 2-fold higher than in the lungs of healthy animals. The tendency of HY to preferentially concentrate in lung metastases, combined with its potent antiproliferative activities, may render HY as a useful supplementary modality in the treatment of metastatic cancer irrespective of photoactivation.

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Cancer is currently viewed as a cell cycle disease. Strategies to treat malignancies are thus shifting from toxic chemotherapy to agents that switch-off positive cell replication transducers specifically or to activators of endogenous negative cell cycle regulators (p53, p21^{waf1}, Kip and INK families of proteins).¹ The feasibility of the novel approach is exemplified by recent success in achieving clinical remissions by Imatinib Mesylate (Gleevec), a rationally designed catalytic domain inhibitor of Abl, c-Kit and PDGFR tyrosine kinases.^{2–4} Other agents such as the cyclin-dependent kinase inhibitors, flavopiridol, 7-hydroxystaurosporine (UCN-01) and staurosporine are at different phases of clinical development.^{5,6}

Additional attractive targets for intracellular signaling inhibition are modulators of the PKC family of isoenzymes. Perihydroxylated perylene quinones constitute a unique class of photoactivated inhibitors of ser/thr kinases. In this group, hypericin (HY) has been reported to inhibit PKC,^{7,8} Erk1/2 kinases and also epidermal growth factor receptor tyrosine kinase.^{9–11} Most of these signal-transduction inhibitory activities of hypericin have been attributed to the photodynamic properties of the compound that were achieved when HY was excited by light at wavelengths absorbed by the molecule.¹¹ However, recent observations suggest that HY, in addition to its light-dependent anticancer activities,¹² possesses potent anticancer activities and ability to inhibit different signal transduction pathways also in the absence of light activation (dark effects).^{13–15}

We recently demonstrated that the newly identified anti-cancer activities of HY in the dark differ from the well-characterized light-induced effects of the compound in kinetics, modes of tumor cell death and their underlying mechanisms.¹³ Cell death induction by HY with light occurs rapidly. Within hours after light irradiation, cells undergo apoptosis, or if the light dose is high, also

necrosis. In the dark, on the other hand, tumor cell exposure to HY is required for at least 48 hr in order to inhibit cell proliferation. At higher HY concentrations (> 10 μ M), the compound killed the tumor cells in the dark *via* mitotic cell death.¹⁶ Mitotic cell death is a process observed in cancer cells following treatment with different chemotherapeutic agents as well as radiotherapy. The phenomenon is characterized by cells accumulation at G₂/M, uncoupling of apoptosis, increased cell volume and multinucleation leading to cell death.¹⁷ Although the precise mechanisms leading to mitotic cell death are not fully understood, we have recently identified processes by which hypericin generates this phenomenon in the absence of light irradiation. Our understanding of this mode of action is summarized in Figure 1.

In vivo, light-independent activities of HY have been shown to translate into inhibition of primary tumor growth associated with significantly prolonged survival of tumor bearing animals.¹³

These findings prompted us to examine the efficacy of HY in inhibiting a most critical aspect of tumorigenesis: development of metastases. It was found that serial HY administrations combined with resection of primary tumors can significantly reduce animal death rates from metastases in 2 different metastatic murine tumor models. In addition, we demonstrate the localization of HY in tumoral tissues as well as in their metastatic lesions. Long-term HY sequestration is more pronounced in tumor lung metastases and may lead to potent anti-metastatic effects. We discuss the possible contribution of protracted presence of HY in lung metastases to the anti-metastatic activities of HY in the absence of photosensitization with light.

MATERIAL AND METHODS

Reagents

Hypericin (10,11-dimethyl-1,3,4,6,8,13-hexahydroxy-naphthodanthrone) was synthesized as described previously.¹⁸ HY was dissolved in 70% aqueous ethanol to a stock solution of 3 mg/ml with subsequent dilutions in sterile double distilled H₂O.

Cell lines and culture conditions

DA3 murine mammary adenocarcinoma cells¹⁹ and SQ2 murine anaplastic squamous cell carcinoma were grown in Dulbecco's MEM medium supplemented with 10% fetal calf serum, 2 mM glutamine and 100 units/ml penicillin-streptomycin (GibcoBRL Life Technologies, Ltd., Paisley, Scotland) at 37°C in a humidified 5% CO₂/95% air atmosphere.

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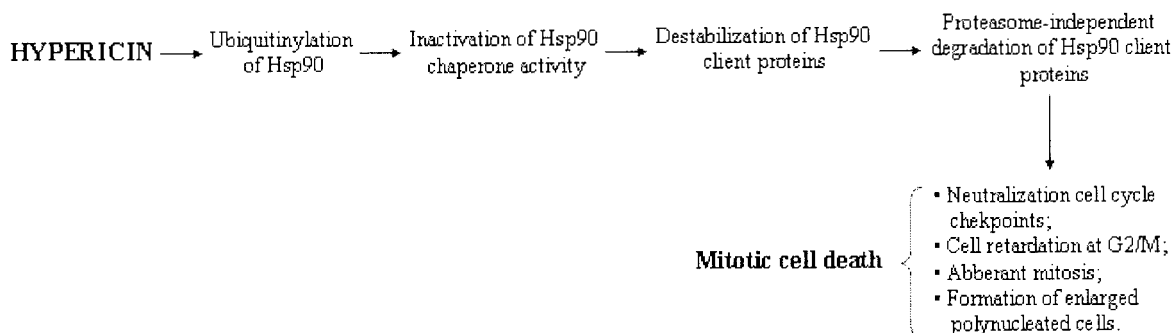


FIGURE 1 – Molecular effects of hypericin in cancer cells that culminate in mitotic cell death.

Animals

BALB/c mice, 9–10 weeks old, were purchased from Harlan, Ltd. (Jerusalem, Israel) grown and maintained on Purina. Males were used in the SQ2 experiments and females in the DA3 experiments. Experiments were conducted in compliance with animal welfare regulations of the ministry of health as approved by the institutional animal care committee.

Generation of tumors and design of experimental therapy

The primary DA3- or SQ2-derived murine tumors were generated by inoculating 5×10^5 tumor cells in 0.2 ml serum-free Dulbecco's medium intradermally into depilated dorsa of the mice. When DA3 tumors reached sizes of 5–6 mm in diameter and SQ2 tumors reached sizes of 7–8 mm, the animals were randomized into 4 experimental groups and injected intraperitoneally (i.p.) either with HY (5 mg/kg) or with vehicle alone. Five days later, as the primary tumors were 7–8 mm in diameter in mice bearing DA3 tumors or 10–12 mm in mice bearing SQ2 carcinoma, the animals were anaesthetized with Ketamine (Parke-Davis, Eastleigh, Hampshire, UK)-Xylazine (Vitamed, Bat-Yam, Israel) mix, 100 mg/kg and 6.25 mg/kg, respectively, and the primary tumors removed by excision. The HY-treated mice received 3–5 doses of HY at 5-day intervals. From the onset of treatments with HY animals were maintained in metal cages with covers that contain 1 cm diameter pores designed to minimize penetration of light and allow sufficient exchange of air. Laboratory lights were shut off upon animal removal from cages and ambient light kept below 0.03 mW/cm^2 (light intensities were quantified using the IL 1350 Radiometer/Photometer, from International Light, Inc., Massachusetts).

Histological studies

Mice bearing DA3 tumor in the dorsum were injected with a single dose of HY (10 mg/kg) i.p., approximately 50 days after tumor cell inoculation. The animals were then maintained in the dark for time intervals ranging between 24–72 hr. At the end of each incubation period the animals were killed, their lungs removed and fixed in 4% buffered formaldehyde (Sigma Chemical Co., St. Louis, MO). The specimens were embedded in paraffin, sections 5 μm in depth prepared and stained with hematoxylin-eosin (H&E), and subjected to morphological examinations.

HY accumulation in murine organs

The concentration of HY in internal organs was analyzed using fiber-optic laser induced fluorescence (LIF) and multipixel spectral imaging (MSI) systems. LIF, which is based on a point measurement of signals from a fluorescence probe (fluorophore) following laser-induced activation of the tissue-sequestered compound,²⁰ was used for quantitative assessment of HY distributed in organs of tumor-free and tumor-bearing (TB) animals. BALB/c mice inoculated with primary DA3 or SQ2 tumors and bearing lung metastases (approximately 50 days post tumor inoculation), were injected with HY (10 mg/kg; i.p.). The animals were maintained in

the dark for time intervals ranging from 3 to 72 hr. At the end of each incubation period the animals were killed, their organs extracted and placed on a plate with attached bifurcated optical fiber (Oriel, Stamford, CT, model 77533). One leg of the fiber was attached to an argon ion laser source (Melles Griot, 43 series), with an output of 10 mW/cm^2 emitting at 477 nm for excitation. The other leg was connected to a spectrofluorophotometer (RF-5301 PC, Shimadzu, Japan) calibrated to detect emission spectrum of fluorophores at 550–750 nm range. Fluorescent intensity emitted by HY from tissue samples was measured in 5–7 different sections of the sample. Background signals, originating from the tissues of animals not administered with HY, were subtracted from the spectra, and fluorescence intensities at 602 nm were used as maxima for pharmacokinetic curves.

Fluorescence images of normal lungs and lungs with metastases, extracted from TB mice injected with HY (10 mg/kg; i.p.), were obtained using multipixel spectral image-analysis system (SpectraCube™ SD300, Applied Spectral Imaging, Migdal Ha'Emek, Israel) as previously described.²¹ Fluorescence was acquired in the spectral range of 550–750 nm at excitation $500 \pm 20 \text{ nm}$.

Statistical analysis

Animal survival data were analyzed using the Logrank/Mantel-Cox test. For *ex-vivo* studies the data was analyzed using a 2-tail Student's *t*-test. *p*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Effects of HY on the growth of DA3 breast carcinoma-derived lung metastases

HY can interfere with the growth of primary tumors;¹³ however, since growth of metastases constitutes the major cause for treatment failure in cancer, the effects of HY on the advanced stages of the disease, when metastases already exist, were examined.

DA3 cell-derived tumors develop metastases predominantly in the lung.¹² The anti-metastatic efficacy of HY was evaluated as animal survival measured up to 300 days. This parameter was chosen because weight differences between lungs bearing metastases and normal lungs in this tumor model were minimal. On occasions the weight of lungs burdened with metastases exceeded lung weight in tumor-free animals, by only 38–45% ($285 \pm 18 \text{ mg}$ vs. $210 \pm 20 \text{ mg}$ in healthy mice) even at advanced stages of the disease.

To focus selectively on the effects of HY on metastases, the primary tumors were surgically resected at a stage, when micro-metastases have already existed. Calibrations determined that excision of primary tumors at a diameter of 8 mm (approximately 15 days post DA3 cell inoculation) result in death rates of approximately 85% of the mice.

The tumor bearing mice were randomized into 4 groups. The first received 6 i.p. HY administrations at declining dosages at

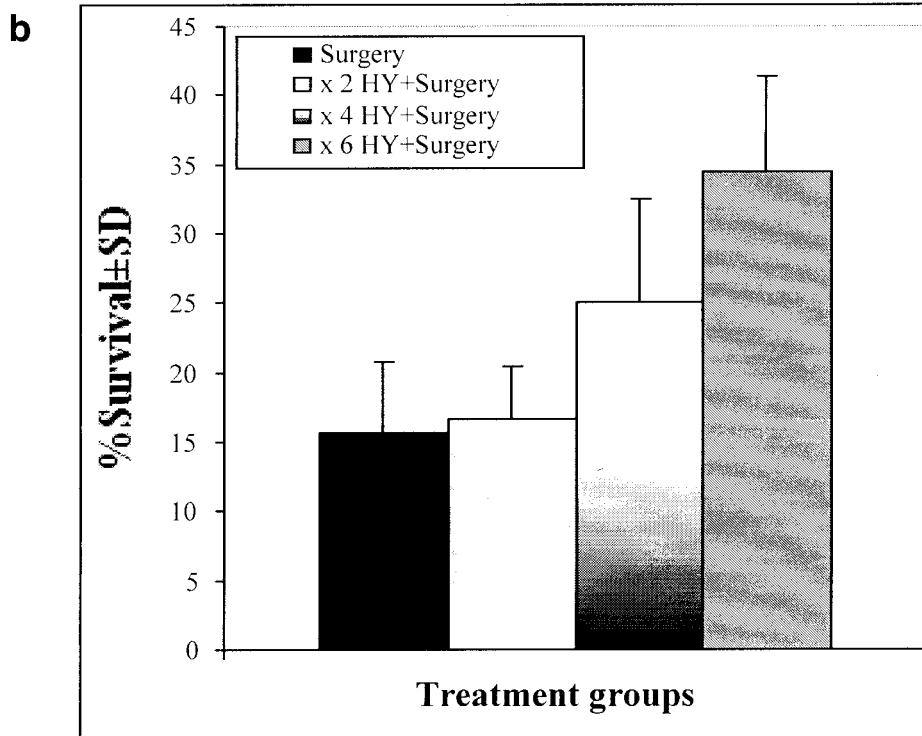
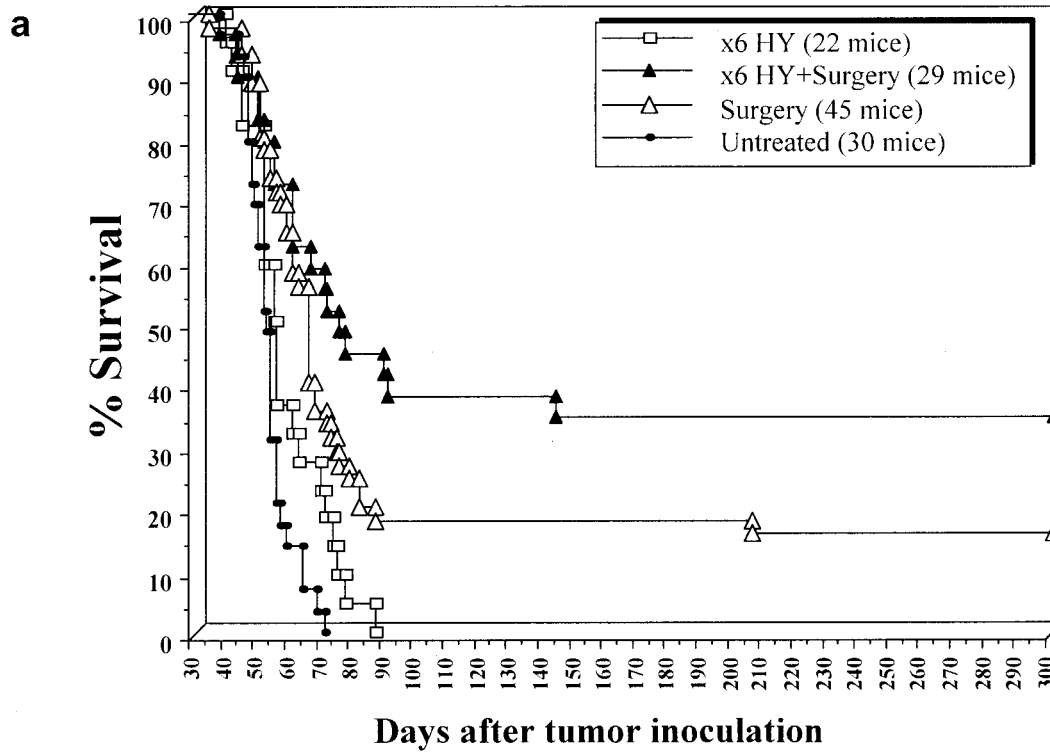


FIGURE 2 – (a) Multiple HY dose regimens combined with surgery protects mice bearing breast adenocarcinoma tumors from death caused by metastases. Mice bearing DA3 tumors (8 ± 1 mm in diameter) were randomized into 4 groups. In 1 group, tumors were excised and 6 i.p. HY administrations at declining dose schedules were given at 5-day intervals, beginning 5 days prior to surgery (x6 HY + Surgery). One control group had the tumors resected (Surgery), a second control group received only treatments with HY (HY) and in a third the DA3 tumors were left intact (Untreated). Animal survival was monitored for 300 days. The figure is a summary of 5 independent experiments (the total number of animals in each group is indicated in the figure). (b) Animals bearing DA3-derived adenocarcinoma were treated with 2, 4 or 6 systemically (i.p.) HY administrations combined with surgical removal of primary tumors. Animal survival was then monitored for 160 days. The results are presented as % Survival + S.D.

5-day intervals between schedules. The first HY dose of 5 mg/kg was initiated 5 days prior to tumor resection. The second dose was 2.5 mg/kg and was followed by 4 maintenance doses of 1.25 mg/kg each. The second treatment group consisted of surgical resection with no HY dosing and the third group received only treatments with HY applied in parallel to the first group. A fourth group consisted of untreated DA3 tumor-bearing mice. The declining HY dose regimens and relatively long intervals between HY administrations were selected due to the long half-life of this compound in rodents (discussed hereunder). Animal survival was monitored for 300 days after tumor cell inoculation.

The results (Fig. 2a) show that combining HY therapy with surgical resection of the primary tumors led to a cure rate of 34.5% of the mice compared to 15.6% cured by surgery alone or 0% survival in untreated group ($p < 0.001$). Although, treatment with HY without tumor resection resulted in death of all mice in the group, a modest prolongation of animal survival from 52 ± 7.2 days in the untreated control group to 58 ± 12.4 days was observed ($p = 0.03$).

Examination of dose-dependent effects of HY on animal cure from metastases in DA3 tumor-excised mice revealed that the cure rates of the treatment increased from 16% following tumor excision to 25% and 35% when primary tumor resection was combined with 4 or 6 regimens of HY, respectively (Fig. 2b). Two dose regimens of HY were insufficient and death rates remained similar to the control groups in which tumor resection was the sole treatment (Fig. 2b).

The effect of HY on lung metastases has been examined histologically. Animals bearing DA3 tumor metastases and administered with a single dose of HY 10 mg/kg i.p were sacrificed after 24, 48 and 72 hr. The lungs were removed, sections prepared and stained with H&E. The histological examination (Fig. 3) revealed complete destruction of several, but not all, of the metastatic foci following treatment with HY. Such destruction became evident only >48 hr after HY administration as shown in Figure 3a taken at the 72 hr time-point. No destruction of metastatic foci could be detected in the untreated control mice (Fig. 3b). These findings suggest that treatment of TB animals with HY can reduce the metastatic load, although a single dose of the compound was insufficient to cure the animals or prolong their survival (data not shown).

Immunological considerations in animal cure from metastatic disease

An attempt was made to test whether mice cured by primary DA3 tumor resection followed by treatment with HY develop immune-mediated resistance to this cell line. Animals that survived DA3 metastases were reinjected with 2.5×10^5 live DA3 cells/mouse. The mean survival time (MST) of mice that have previously been cured with 6 dose schedules of HY and primary tumor excision (62.4 ± 13.4 days) did not differ significantly from the MST of naïve mice inoculated with DA3 cells (59.5 ± 5.9 days), or from mice subjected to tumor excision alone (63.4 ± 12.3). The data does not support development of any significant anti-tumoral protective activity in animals previously cured with HY.

Anti-metastatic effect of HY on SQ2 murine squamous cell carcinoma-derived metastases

In order to corroborate observations in the breast carcinoma tumor model, which show improved cure rates from metastatic disease by treatment with HY, our studies were extended to analysis of effects on metastases in a squamous cell carcinoma tumor model. Calibration studies indicated that excision of primary SQ2 cell-derived tumors at a diameter of 10–12 mm, resulted in metastases-related death of approximately 80–85% of the animals. Effects of HY on SQ2 metastatic development was examined following primary tumor excision as they at-

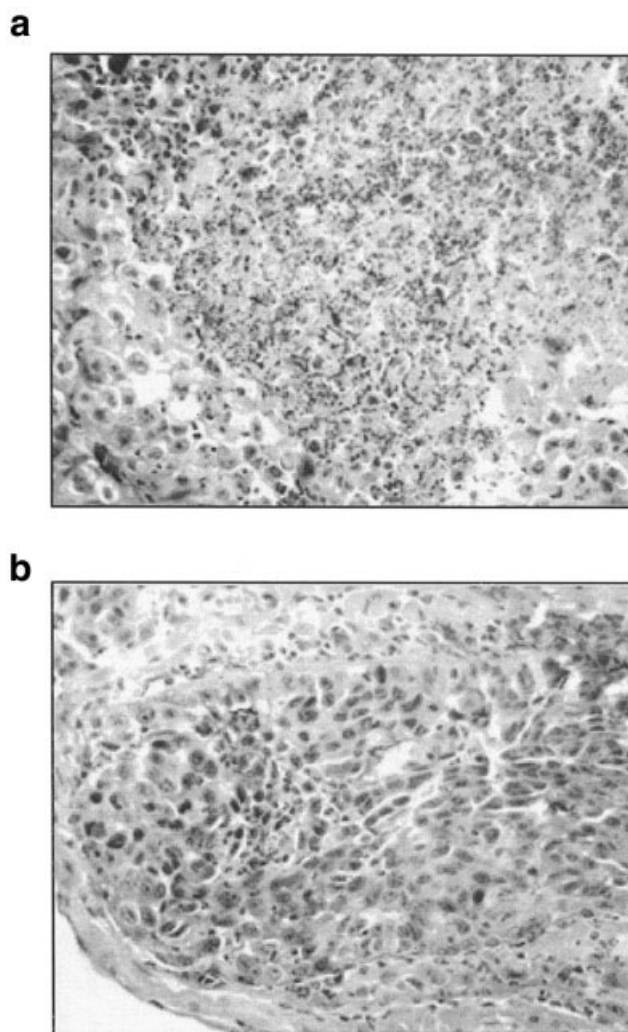


FIGURE 3 – Destruction of DA3 tumor lung metastases by HY. Mice bearing lung metastases of DA3 tumors were administered with a single dose of HY (10 mg/kg) i.p. The animals were sacrificed 72 hr later and their lungs removed, fixed and embedded in paraffin. Sections were prepared and stained with H&E. (a) Metastatic focus which underwent massive destruction following HY treatment. (b) Untreated (control) lung with metastases.

tained these diameters. One dose of HY, 5 mg/kg, was administered followed by 1 lower dose of 2.5 mg/kg and 3 maintenance doses of 1.25 mg/kg each at 5-day intervals beginning 5 days prior to primary tumor excision (a total of 5 doses of HY). The results (Fig. 4) show that animal treatment with HY following tumor excision prevented the death of 46.1% of the mice compared to 17.7% in the primary tumor-resected control group. Median animal survival time was prolonged from 52.5 days in the untreated group to 97 days in the resected group to 190 days in the combination treatment group ($p < 0.001$ vs. the untreated control group).

These findings indicate that treatment of mice bearing DA3 or SQ2 tumor metastases with multiple-dose schedules of HY can prevent animal death when administered shortly after primary tumor removal at a stage in which tumor micrometastases already exist in the lungs. The net protective rates of this treatment (vs. tumor excision alone) in mice bearing breast adenocarcinoma was approximately 19% and in mice with squamous cell carcinoma ~28%.

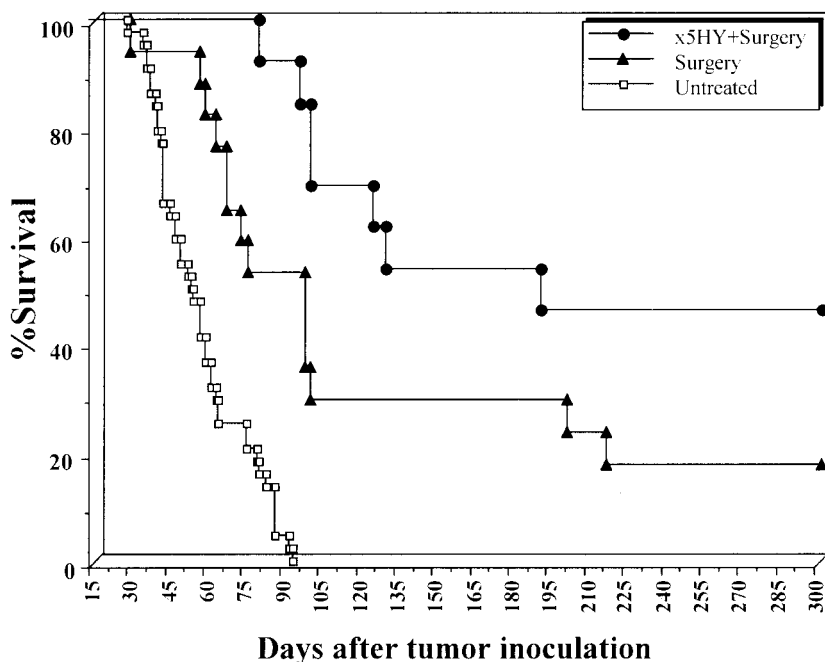


FIGURE 4—Prevention of metastases-induced animal death in mice bearing SQ2-derived squamous cell carcinoma by HY following primary tumor removal by surgery. Mice bearing squamous cell carcinoma tumors were treated with 5 consecutive HY administrations at 5-day intervals combined with primary tumor resection at diameters of 10–12 mm (x5 HY+Surgery). Untreated animals as well as mice treated with surgery alone (Surgery) served as controls. Animal survival was monitored for 300 days.

Distribution of HY in organs and tissues of TB and healthy animals

The potent anti-metastatic activities of HY, which occur in the dark, prompted analysis of the correlation between the actual presence of this compound at the metastatic site and its antitumoral activity. The biodistribution of HY in several murine organs and tissues of tumor bearing mice, including lungs heavily loaded with metastases, was examined in comparison to organs obtained from healthy animals.

Mice bearing DA3 and SQ2 primary tumors that developed lung metastases were administered with HY (10 mg/kg, i.p.) and maintained for time intervals ranging from 3 to 96 hr. At the end of each incubation period, the animals were sacrificed, their organs extracted and the content of HY in the organs measured using the LIF technique as described in Methods. HY was found to sequester predominantly in the primary tumors and in metastatic lungs in both DA3 and SQ2 tumors (data not shown). Although HY was also found in other organs such as liver, spleen, kidney and in muscle tissue, the compound was cleared from these organs by 24 hr. Significant retention of HY after this time-point was noted only in the tumor and in the lungs. Comparison of the amounts of HY sequestered in normal (tumor-free) vs. metastases-burdened lungs, revealed a 1.8-fold higher concentration of the compound in the DA3 lung metastases, 48 hr post HY administration (Fig. 5a), and a 4-fold higher concentration in the squamous cell carcinoma-derived metastases after 72 hr (Fig. 5b).

Accumulation of HY in metastatic foci in the lungs was also monitored using multipixel spectral image-analysis system (MSI). This technique combines conventional imaging with spectroscopy and allows visualization of HY fluorescence in each pixel of interferogram of the image.

Mice bearing DA3 lung metastases as well as normal animals were administered with 10 mg/kg HY. Twenty-four hours later the animals were killed, their lungs extracted and subjected to MSI analysis as described in Methods. Figure 6 shows normal (red-green-blue [RGB]) (Fig. 6a,c) and fluorescence images (Fig. 6b,d) of tumor-free lungs and lungs bearing a high load of DA3 adenocarcinoma metastases. In normal lungs (Fig. 6a,b), some HY could be detected within the lung tissue; however, in lungs bearing metastases, larger HY amounts were found in the lungs preferentially confined to metastatic foci (Fig. 6c,d).

These observations suggest that in tumor-bearing mice HY accumulates preferentially in the primary tumors and metastatic foci. Our results also show that retention of HY in squamous tumor lung metastases was longer than in breast tumor-derived metastases.

DISCUSSION

The unique ability of the photodynamic dianthraquinone hypericin to exert biological activities in the dark is shown here to translate into potent anti-metastatic activity. Since HY is a powerful photodynamic agent most of its anticancer activities have been correlated with the effects of light. Indeed, several investigators have reported absence of HY-mediated cytotoxicity in the dark even at high hypericin concentrations.²² These observations were noted when cell exposure to HY was limited to periods of several hours. Our studies demonstrate that the effects of HY in the dark have significantly different kinetics, requiring longer exposures of cancerous cells to HY (for up to 72 hr) in the dark. These conditions reveal the existence of light-independent antitumoral effects of HY, which can be observed in tumor cell models *in vitro*^{13,14} as well as in animal models.¹³ The mode of cell death that is induced in the dark also differs from the phototoxic activities. While the light-mediated photodynamic effects of HY lead to apoptosis at low light doses and to necrosis at higher light doses,¹⁸ the dark effects have been found to elicit mitotic cell death.¹⁶ A snowball process of disrupting cell cycle signaling pathways in tumor cells has been found to begin with ubiquitinylation of the Hsp90 chaperone. Chaperone function is abrogated and a multitude of Hsp90 client proteins are destabilized and concomitantly degraded. The loss of key signaling components culminates in mitotic death.¹⁶

The biochemical basis for the observed antitumoral activities of HY in the dark appears to be the relatively low red/ox potential of this molecule, $E_1/V = -1.012$ electron volt, measured by electron spin resonance.²³ It may be low enough to enable HY to function as an acceptor from cellular electron transfer reactions and high enough to generate active oxygen species. Indeed, electrochemical studies show that HY has electron accepting as well as electron donating properties and can function as both oxidizing and reducing agents that facilitate the activities in the dark.²⁴

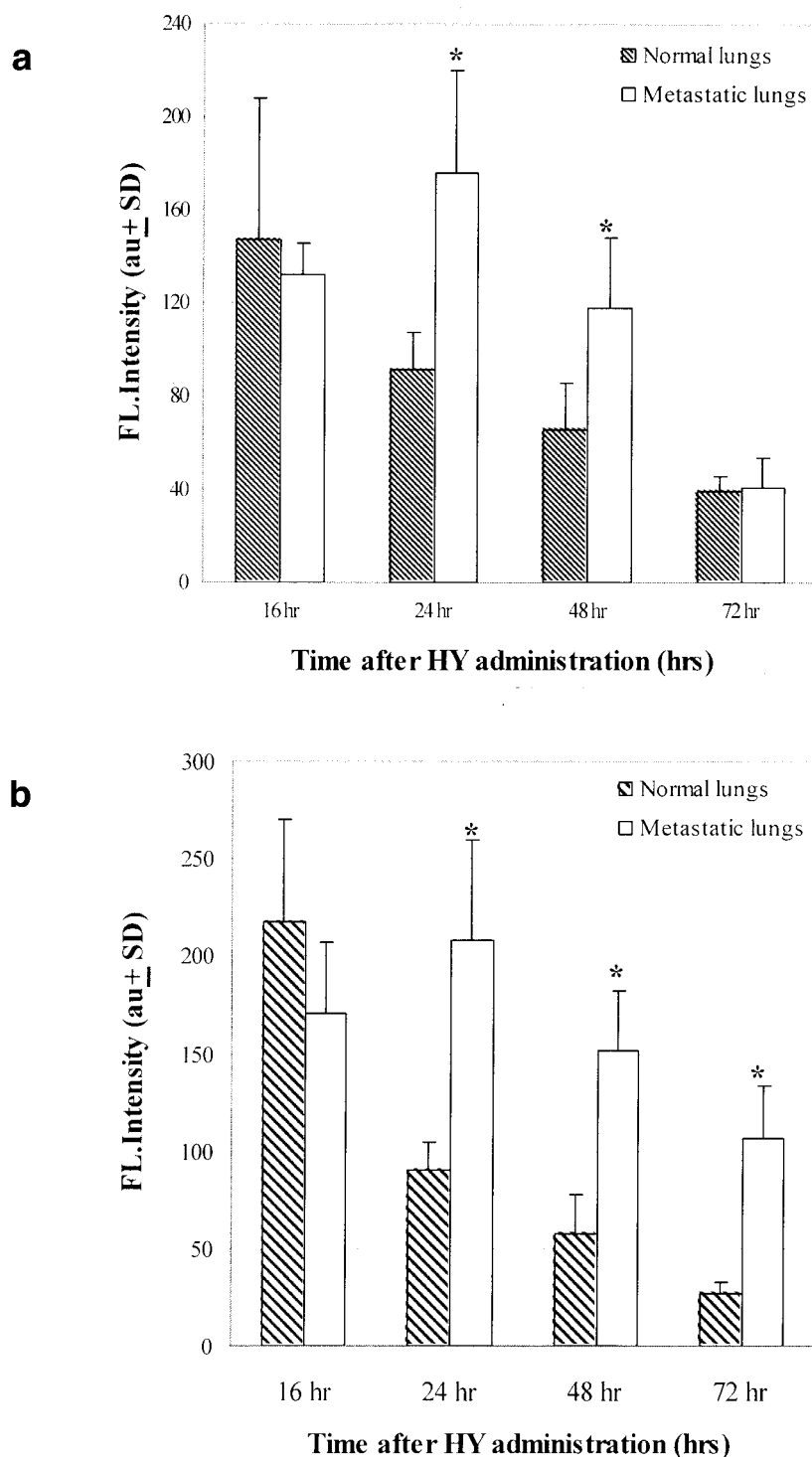


FIGURE 5 – Comparative analysis of HY accumulation in normal or metastatic lungs. Mice bearing DA3- (*a*) and SQ2-derived lung metastases (*b*) were administered with HY (10 mg/kg, i.p.). HY accumulation in normal lungs, obtained from tumor-free animals, and in lungs from mice bearing abundant metastases was measured using LIF technique, 16–72 hr after HY administration. The results are presented as Mean \pm S.D. of fluorescence intensity emitted by HY from lungs measured in 5–7 different sections of the sample. The data present a summary of 2 independent experiments utilizing 3–4 mice in each group.

Since metastases constitute the main obstacle for successful anticancer therapy, ability to cure early metastatic disease can significantly broaden the therapeutic scope of HY. Using 2 murine metastatic cancer models, we have identified several characteristics of the anti-metastatic activities of this molecule. One important feature is the ability to extend animal survival beyond 300 days, which can be viewed as actual cure of metastatic disease by a relatively small number of infrequent dosing schedules (up to 6 schedules applied at 5-day intervals). Focus on metastasis was achieved by surgical primary tumor excision at a stage in which

micrometastases already exist. HY treatment at this stage was found to cure approximately 35% and 46% of animals, which bore adenocarcinoma (Fig. 2) or squamous cell carcinoma metastases (Fig. 4), respectively. Maximal effect in DA3 adenocarcinoma bearing mice was observed following 6 dosing schedules with HY (Fig. 2), while 5 doses of HY were sufficient to cure 46% of the mice bearing SQ2 tumors (Fig. 4). Although endogenous differences in the sensitivity of the cells from the 2 tumors to hypericin may exist, at least part of the higher anti-metastatic efficacy of HY in the squamous carcinoma model may be attributed to a longer

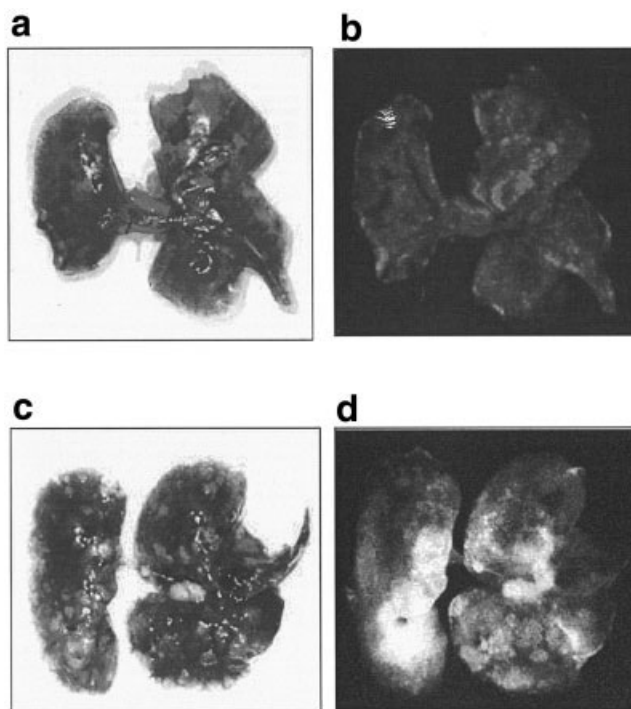


FIGURE 6—Multipixel spectral imaging (MSI) analysis of HY accumulation in normal and metastatic lungs. Mice bearing DA3 tumor metastases in the lungs as well tumor-free (normal) animals were injected with HY (10 mg/kg, i.p.) Twenty-four hours later their lungs were extracted and photographed using multipixel spectral image-analysis system given red fluorescence due to HY emission (excitation at 500 ± 20 nm). (a,b) RGB (red-green-blue) and fluorescence images of normal lungs, respectively. (c,d) RGB (red-green-blue) and fluorescence images of lungs containing widespread DA3 adenocarcinoma metastases, respectively.

retention of the compound in metastatic foci of this tumor. For example, at 72 hr, HY retention in SQ2 lung metastases was 3-fold higher than in normal lungs, whereas in DA3 affected lungs HY content equated normal lung levels (Fig. 5), suggesting clearance from DA3 tumor foci at this time-point.

Treatment of animals bearing early DA3 tumor metastases with HY yields better efficacies compared to treatment of animals bearing primary tumors.¹³ Although, the mean survival time of primary tumor-bearing animals treated with HY was significantly prolonged compared to untreated control mice, these animals eventually died.¹³ In contrast, considerable portions of animals in which the primary tumors were resected could be cured. Thus, in the absence of photosensitization, HY is unlikely to provide effective protection against primary tumors bearing larger tumor loads.

In mice cured from metastatic disease by treatment with HY and subsequently challenged with live tumor cells, we could find no evidence for development of anti-tumoral immune responses. Thus, induction of metastatic regression by the treatment with HY appears to be confined to direct cytotoxic effects of this molecule to cancerous cells in metastatic lesions and not to immunological responses. Indeed, HY tissue fluorescence analyses and histological studies provide evidence for selective accumulation of HY in lung metastatic foci (Fig. 6) and for their destruction *in vivo* (Fig. 3).

HY has reached phase II clinical trials as a treatment modality for malignant glioma.²⁵ The new findings described here suggest that this compound may serve as a broad range anti-metastatic agent.

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REFERENCES

- Shapiro GI, Harper JW. Anticancer drug targets: cell cycle and checkpoints control. *J Clin Invest* 1999;104:1645–53.
- Joensuu H, Dimitrijevic S. Tyrosine kinase inhibitor imatinib (STI571) as an anticancer agent for solid tumours. *Ann Med* 2001; 33:451–5.
- Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* 2002;346:645–52.
- Tuveson DA, Willis NA, Jacks T, Griffin JD, Singer S, Fletcher CD, Demetri GD. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene* 2001;20:5054–8.
- Senderowicz AM. Small molecule modulators of cyclin-dependent kinases for cancer therapy. *Oncogene* 2000;19:6600–6.
- Schnier JB, Nishi K, Goodrich DW, Bradbury EM. G1 arrest and down-regulation of cyclin E/cyclin-dependent kinase-2 by the protein kinase inhibitor staurosporine are dependent on the retinoblastoma protein in the bladder carcinoma cell line 5637. *Proc Natl Acad Sci U S A* 1996;93:5941–6.
- Diwu Z, Zimmermann J, Meyer Th, Lawn JW. Design, synthesis, and investigation of mechanisms of action of novel protein kinase C inhibitors: perylene quinonoid pigments. *Biochem Pharmacol* 1994; 47:373–85.
- 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* 1989;165:1207–12.
- Agostinis P, Vandenbogaerde A, Donella-Deana A, Pinna LA, Lee KT, Goris J, Merlevede W, Vandenheede JR, De Witte P. Photosensitized inhibition of growth factor-regulated protein kinases by hypericin. *Biochem Pharmacol* 1995;49:1615–22.
- A comparative analysis of the photosensitized inhibition of growth-factor regulated protein kinases by hypericin-derivatives. *Biochem Biophys Res Commun* 1996;220:613–7.
- Agostinis P, Assefa Z, Vantiegheem A, Vandenheede JR, Merlevede W, De Witte P. Apoptotic and anti-apoptotic signaling pathways induced by photodynamic therapy with hypericin. *Adv Enzyme Regul* 2000;40:157–82.
- Blank M, Lavie G, Mandel M, Keisari Y. Effects of photodynamic therapy with hypericin in mice bearing highly invasive solid tumors. *Oncol Res* 2001;12:409–18.
- Blank M, Mandel M, Hazan S, Keisari Y, Lavie G. Anti-cancer activities of hypericin in the dark. *Photochem Photobiol* 2001;74: 120–5.
- Hwang MS, Yum YN, Joo JH, Kim S, Lee KK, Gee SW, Kang HI, Kim OH. Inhibition of c-erbB-2 expression and activity in human ovarian carcinoma cells by hypericin. *Anticancer Res* 2001;21:2649–55.
- Hostanska K, Reichling J, Bommer S, Weber M, Saller R. Aqueous ethanolic extract of St. John's wort (*Hypericum perforatum* L.) induces growth inhibition and apoptosis in human malignant cells in vitro. *Pharmazie* 2002;57:323–31.
- Blank M, Mandel M, Keisari Y, Meruelo D, Lavie G. Enhanced ubiquitinylation of Hsp90 as a potential mechanism for mitotic cell death in cancer cells induced with Hypericin. *Cancer Res* 2003;63: 8241–47.
- Erenpreisa J, Cragg MS. Mitotic death: a mechanism of survival? *Cancer Cell Internat* 2001;1:1–7.
- Lavie G, Kaplinsky C, Toren A, Aizman I, Meruelo D, Mazur Y, Mandel M. A photodynamic pathway to apoptosis and necrosis induced by dimethyl tetrahydroxyhelianthone and hypericin in leukemic cells: possible relevance to photodynamic therapy. *Br J Cancer* 1999;79:423–32.
- Sotomayor EM, Fu YX, Lopez-Cepero M, Herbert L, Jimenez JJ, Albarracin C, Lopez DM. Role of tumor-derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. II. Down-regulation of macrophage-mediated cytotoxicity by tumor-derived granulocyte-macrophage colony-stimulating factor. *J Immunol* 1991;147:2861–73.

20. Malik Z, Kostenich G, Roitman L, Orenstein, A. Topical application of 5-aminolevulinic acid, DMSO and EDTA: protoporphyrin IX accumulation in skin and tumours of mice. *J. Photochem Photobiol B: Biology* 1995;25:213–8.
21. Orenstein A, Kostenich G, Rothmann C, Barshack I, Malik, Z. Imaging of human skin lesions using Multipixel Fourier Transform Spectroscopy. *Lasers Med Sci* 1998;13:112–8.
22. Vandenberghe AL, Cuveele JF, Proot P, Himpens BE, Merlevede WJ, de Witte PA. Differential cytotoxic effects induced after photosensitization by hypericin. *J Photochem Photobiol B* 1997;38:136–42.
23. Gerson F, Gescheidt G, Haering P, Mazur Y, Freeman D, Spreitzer H, Daub G. Electron acceptor properties of hypericin and its salts: an ESR/ENDOR and electrochemical study. *J Am Chem Soc* 1995;117:11861–6.
24. Redepenning J, Tao N. Measurement of formal potentials for hypericin in dimethyl sulfoxide. *Photochem Photobiol* 1993;58:532–5.
25. Couldwell WT, Gopalakrishna R, Hinton DR, He S, Weiss MH, Law RE, Apuzzo ML, Law RE. Hypericin: a potential antiglioma therapy. *Neurosurgery* 1994;35:705–10.