Hypericin targets multiple signaling mediators in cancer cells generating unique, anti-tumoral, anti-metastatic, and anti-angiogenic activities with evidence for clinical applicability

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ABSTRACT

The goals of this review are to comprehensively analyze the diverse biological activities displayed by a most potent photodynamic agent – hypericin. Hypericin is a lipophilic redox-reactive molecule possessing a redox potential low enough to act as electron acceptor, subsequently discharging these electrons to oxygen, generating ROS. This property enables intracellular hypericin to retain redox activities in the dark. In cells, hypericin sequesters in endoplasmic reticulum and Golgi apparatus membranes and photo-oxidizes membranal lipoproteins. However, the most relevant cytosolic hypericin target is the Hsp90 chaperone. We have shown that hypericin selectively binds to and oxidizes Hsp90, inducing its forced polyubiquitinylation, functional inactivation and rapid degradation in a proteasome independent manner. Hsp90 physiological association with the myriad of client proteins is disrupted and several signaling mediators, cell cycling and proliferation regulators are destabilized and degraded. Cell cycle checkpoints affected secondarily cause uneven, premature mitosis, (karyokinesis with no cytokinesis), forming polykaryonic giant cells, hallmark of mitotic catastrophe also known as mitotic cell death. HIF-1α, the master regulator of VEGF synthesis and angiogenesis inducer is also an Hsp90 client protein. HIF-1α is physiologically degraded by oxygen but also by the hypericin-induced Hsp90 ablation, inducing potent tumor neoangiogenesis inhibition. Hsp90 is implicated in mediating inheritable epigenetic modifications, causing epigenetic signature changes in key developmentally regulated genes and tumor cell exit from proliferation cycles. Expression of EZH2, the Polycomb repressor complex-2 catalytic subunit, which trimethylates histone H3lys27 is suppressed, class-I HDACs expression downregulated and HDAC1-Dnmt1-EZH2 complex formations diminish. Deficiencies in HDACs cellular contents lead to histones H3 and H4 hyperacetylation which together with diminished H3K27-trimethylation relax chromatin structure, activating transcription including differentiation-promoting genes. In GBM cells, neuroglial differentiation antigens become expressed, cytoarchitecture modulated and the cells undergo tumor cell differentiation. Indeed, clinically significant anti-GBM effects were obtained in a clinical trial in recurrent, progressive GBM patients.

Key Words: Epigenetics, Anti-angiogenic, Cancer, Glioblastoma, Hypericin, PRC2, EZH2, Histone deacetylases, redox, Hsp90, Ubiquitin

1. Background

Current trends in molecular anticancer research continue to aim for identification of organic molecules which can interfere with cell proliferation signaling networks and thereby elicit tumor cell cycle arrest. Proliferation arrest which follows primarily genotoxic stress can direct cancerous cells to undergo programmed cell death (PCD) via one of several forms of PCD. The primary form is apoptosis, however solid tumors refractory to apoptosis have been found to undergo necroptosis (a caspase-independent programmed necrosis) (*Linkermann, A, et al. (2014), The New England J. of Med. 370: 455–465; Vanden Berghe, T. et al. (2014), Nature reviews Molecular Cell Biol. 15: 135–147*), autophagic cell death (*Bursch W. (2001), Cell Death Differ. 8: 569–581*) or mitotic cell death, often referred to as mitotic catastrophe (*Blank M., et al. (2007), Cell Cycle 6, 686-695*). The process leading to necroptosis follows a program which differs from apoptosis. Necroptosis is initiated by TNFα stimuli engaging the TNF receptor which signals recruitment of the TNF receptor-associated death domain (TRADD). A suicidal cascade is then initiated, culminating in formation of the necrosome, causing membrane disruption and cell death (*Wang, H. et al. (2014), Molecular Cell. 54: 133–146*).

Mitotic cell death (MCD) mostly occurs following extensive DNA damage which causes defective G2/M checkpoint, leading to premature mitosis. One hallmark of premature mitosis is induction of uneven chromatin condensation (*Roninson IB, et al. (2001) Drug Resist Update 4: 303-313; Chan TA, et al. (2000), Genes Dev. 14:1584-1588*). Cells proceed through aberrant anaphase and telophase without going through cytokinesis, resulting in formation of multimicronucleated polykarion cells, which are another hallmark of mitotic cell death (*Nitta M. et al. (2004), Oncogene 23: 6548-6558; Chan TA, et al. (2000), Genes Dev; 14:1584-1588*).

Cancer cells are intrinsically blocked from undergoing terminal differentiation. Thus, an alternative pathway for neoplastic cells inhibited from perpetual replication cycling is to overcome this block and undergo cell differentiation. Factors which can promote differentiation in solid tumor cells are those which can modify cancer cell gene expression patterns including the aberrations in the epigenetic code which form the neoplasia-promoting platforms. These involve, among others the hypoacetylation of histones H3 and H4 which generate highly condensed cancer cell chromatin found in many tumor types. Acetylation of histones caused by a number of histone acetyl transferases (HATs) and the balancing deacetylation by histone deacetylases (HDACs) play important roles in coordinating gene expression and cell-cycle progression: histone acetylases by relaxing chromatin and increasing the accessibility of transcription factors to chromatin templates in promoter regions of target genes (*Ogryzko VV, et al. (1996), Cell 87: 953-959; Struhl K. (1998), Genes and Dev. 12, 599-606*) and HDACs which counteract this effect and deacetylate histones (*Struhl K. (1998), Genes and Dev. 12, 599-606*).

Reagents which have been found effective in overcoming the differentiation block in tumor cells and which can promote cancer cell differentiation in cultures are the

histone deacetylase inhibitors (HDACi), (*Timmermann S, et al. (2001), Cell Mol Life Sci. 58: 728–736; Göttlicher M, et al. (2001), EMBO J. 20: 6969-6978*). HDACi increase histone acetylation thereby relaxing the highly compacted chromatin in cancer cells. The reduced chromatin compaction increases accessibility of transcription factors to promoters which regulate differentiation and cell cycle checkpoints related genes, enabling transcription and expression of such anti-cancer genes (*Mack GS. (2006), J. Natl Cancer Inst 98: 1443-1444; Bernstein BE, et al. (2007), Cell 128: 669–681*). Since HDACs are elevated in many cancer cell types and are associated with negative prognostic implications in various tumor types (*Minamiya Y, et al. (2011), Lung Cancer 74: 300–304; Weichert W, et al. (2008). Br J Cancer. 98: 604–610*), efforts have been directed to identification of new HDACi for development as anti-cancer drugs and their evaluation in clinical trials as anticancer therapy. Two such HDACi, vorinostat and romidepsin have already been approved by FDA for use in the clinic.

Two fundamental classes of cell proliferation inhibitors can be distinguished based on active or passive functionalities. Passive inhibitors bind to active sites on mediators of signaling cascades, blocking these sites and thereby interfering with physiological ligand interactions, causing functional arrest of the pathway. Hundreds of such small molecules have been isolated from natural origins or synthesized via rational drug design based on three-dimensional molecular structure complementarities. Few examples for the latter are the Abelson tyrosine kinase inhibitor Imatinib mesylate, used to halt chronic myelogenous leukemia (*Schindler T, et al. (2000), Science 289:1938-1942*) and GIST, the second generation drug Nilotinib developed to treat Imatinib resistant patients (*Breccia M, et al. (2010), Leukemia res. 34: 129–134*), the EGFR inhibitor Erlotinib used in non-small cell lung cancer, pancreatic cancer and polycythemia vera (*Kobayashi K, et al. (2013), Targeted Oncol. 8: 27–33)*, oral anticoagulants, inhibitors of HIV virus protease and integrase which inhibit virus replication and many more.

A second class of cell proliferation inhibitors are the active inhibitors which function by actively modifying their targets, via oxidative excitations, phosphorylations, methylations etc. Cancer cells normally exhibit higher basal levels of reactive oxygen species (ROS) compared to normal cells and at the same time display a lower tolerance to increased levels of ROS compared to normal cells (*Gorrini C, et al. (2013), Nat. Rev. Drug Discov. 12: 931–947*). This narrower window of tolerance for oxidative stress has the potential of being exploited as anticancer therapy by utilization of selected redox reagents which can induce oxidative stress at levels exceeding tumor cell tolerance (*Kong Q, et al. (2000), Med. Hypotheses 55: 29–35*).

Important redox reactive molecules in the group of active cell proliferation inhibitors are the photodynamic agents. Photosensitizers are well known to elicit effective anti-cancer activities; however, these are light-dependent actions (at wavelengths absorbed by the photosensitizer) which have primarily been confined to superficial tumors at sites accessible to the activating light source (*Zhao B, et al. (2010), Expert Rev. Anticancer Ther. 10: 1797–1809*). The

average skin penetration of topical photosensitizers is up to 4 millimeters.

Magnitudes of photo-cytotoxicity reactions are determined by concentration of the photosensitizer, the light fluency (duration of irradiation and intensity) and oxygen. Yet, the most important determinant is the efficiency in which a sensitizer utilizes light energy to convert ground state oxygen to its excited, reactive singlet state $O_2(^1\Delta g)$. This property, endogenous to each photosensitizer, is defined as the singlet oxygen quantum yield ($\varphi\Delta$) and it determines the concentration-durations of light irradiation required for a photosensitizer to reach lethal toxicity levels. The higher the quantum yield the least time and lower concentrations are required to cause cell death via apoptosis (or necrosis) in photodynamic therapy (PDT).

As the photo-oxidative burden is further increased, cellular apoptotic machineries become severely damaged due to denaturation of caspases, of the DNA fragmenting endonuclease and granzyme B, disabling their functionalities. Phospholipids in cell membranes are oxidized leading to loss of membrane plasticity, formation of membranous blebs and nuclear swelling which culminate in cell death through unregulated necrosis. In addition to causing direct cancer cell mortality, the tumor-supplying neovasculature is also a major target for photosensitizers. Photosensitized vessels undergo photo-occlusion disrupting the tumor blood supply thereby playing a pivotal role in prevention of tumor growth and spread (*Olivo M, Chin W. (2006), J Environ Pathol Toxicol Oncol. 25:223-237*). The preference of vascular or tumor cell targeting is highly dependent on the relative distribution of the photosensitizer in the cellular versus vascular compartments and can be regulated through pharmacokinetic-based photosensitizer distributions (*Weinberger D, et al. (2005), Current Eye Res., 30: 1–9*).

Although the biological activities of photosensitizers are strictly light-dependent, they are basically redox reactive compounds whose activities are governed by their redox potential. When the redox potential of a compound is lower than that of cytochrome C oxidase - the last player in the cellular ATP-generating oxidative phosphorylation chain in the mitochondrial matrix, the photosensitizer becomes capable of competing with cytochrome C oxidase as an electron acceptor, enabling its activation and endowing it with a multiplicity of biological activities in a light-independent manner (in the dark).

2. Chemical and biological properties of Hypericin

The ultimate goal of this review is to provide a comprehensive analysis of the large array of biological activities displayed by one of the most unique photodynamic and redox reactive molecules found in nature – hypericin (HYP). HYP - (4,5,7,4',7'-hexahydroxy-2,2'-dimethyl-meso- naphtha-dianthrone) is a lipophilic highly potent type II photosensitizer of biological systems. The molecule is found in nature in several species of the genus Hypericum, primarily Hypericum perforatum, where it appears as K^+ monobasic salt and in most laboratory studies is produced synthetically from emodin as Na^+ salt (*Senthil V, et al. (1994), Photochem. & Photobiol., 59, 40-47*). HYP is soluble in polar organic solvents in which

light at absorbed wavelengths photo-activates the molecule.

HYP is among the most potent photodynamic anti-cancer agents. In the visible light range HYP has two absorption peaks in ethanol at 548 and 590 nmeters, with mirror image emission peaks occurring at 642 and 594 nmeters, respectively. HYP is also fluorescent, emitting at λ_{max} = 600 nm (*Yamazaki T, et al. (1993), J. Phys. Chem.* 97*: 7870–7875*).

HYP excites oxygen to form several types of reactive oxygen species (ROS). These include singlet oxygen ($^1O_2$) at high quantum yields, as well as highly reactive superoxides (O_2-.) as noted in spin trap paramagnetic resonance experiments (*Weiner, L. et al. (1992), J Chem Soc., Perkin Trans. 2: 1439–1442*) and also peroxides. The formation of free radical anions O_2^- and hypericinium, suggest engagement of type I reactions as well (*Redepenning J, et al. (1993), Photochem Photobiol. 58: 532-535*). HYP can also induce light dependent acidification of its immediate environment (*Fehr MJ, et al. (1995), J. Am Chem Soc. 117: 18336*), can cause reduction in intracellular pH by proton transfer to surrounding molecules (*Sureau, F, et al. (1996), J Am Chem Soc. 118: 9484–9487*) leading to pH-dependent structural changes in proteins (*Tcherkasskaya, O., et al. (2001), Proteins, 44: 244–254*). HYP can perform uptake of an electron in the absence of oxygen, leading to the formation of semiquinone-type radicals in the presence or absence of light irradiation (*Gerson, F, et al. (1995), J Am Chem Soc. 117, 1861–1865*).

A most unique property of HYP is redox reactivity in the dark when the compound is found within biological systems i.e. cells. The dark activities may be attributed to the relatively low redox potential of HYP, E1/V= -1.012 eV, measured by electron spin resonance (*Gerson, F, et al. (1995), J Am Chem Soc. 117, 1861–1865*). These redox properties enable HYP to act both as reducing and oxidizing agent. The redox potential is low enough to allow the molecule to function as an acceptor from physiological electron transfer reactions and high enough to discharge electrons and generate ROS. It renders HYP useful in deep tissue therapies *in vivo* where accessibility to light is virtually nonexistent. The dark activities however, are less potent compared to the photoactivated toxicities.

HYP is a lipophilic molecule. As such it circulates in the blood bound to lipid shells of lipoproteins, primarily high density (HDL) and low density (LDL) lipoproteins but is also associated with serum albumin as its main carrier. Lipophilicity endows HYP with affinity to cell membranes. Indeed, in cells HYP accumulates in membranes of the endoplasmic reticulum (ER), the Golgi apparatus, lysosomes and mitochondria. (*Agostinis P, et al. (2002), Int. J. Biochem. Cell Biol. 34, 221–241; Ali S. et al. (2002), Int. J. Oncol 21, 531–540*), (and Fig. 1). Buytaert E. and co-workers have shown that the distribution of HYP in membrane encapsulated organelles plays a pivotal role in the photodynamic induction of tumor cell apoptosis by HYP. HYP association with the ER sensitizes the ER under PDT leading to an immediate loss of the sarco/endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) protein, disrupting Ca^{2+} homeostasis and thus causing cell death (*Buytaert E, et al. (2006), FASEB J. 20, 756–758*).

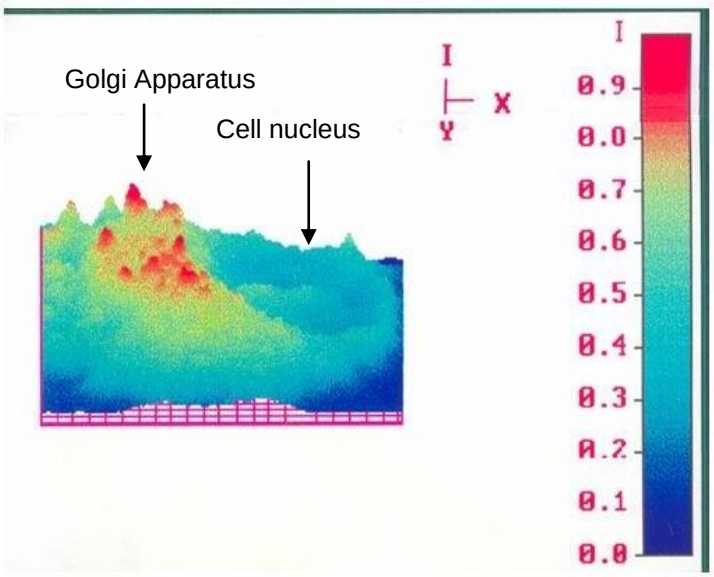

Figure 1. The intracellular distribution of Hypericin in U-251MG human glioblastoma cell line 24 hrs after compound administration (1 μM).

3. Anticancer activities of Hypericin-mediated photodynamic therapy

In cancer bearing organisms, HYP displays preference and accumulates to higher concentrations in tumor cells (*Noell S, et al. (2011), Int. J. Oncol. 38, 1343–1348*).

HYP has been extensively evaluated and for some indications also used in photodynamic therapy (PDT), combining tissue photosensitization and light (at absorbed wavelengths) in the presence of oxygen.

Following photosensitization HYP can cause tumor cell apoptosis (*Ali SM, et al. (2002), Int J Oncol. 21: 531-540; Ali SM, et al. (2003), Int J Oncol. 22: 1181-1191, Review*). The phototoxic effects of HYP cause release of lysosomal enzymes into the cytosol activating caspases- 8, 9, 3, 6 and 7 sequentially. This leads to extensive protein degradation, culminating in apoptosis (*Ali SM, et al. (2001), Int J Mol Med. 8: 521-530*). At higher light fluencies the phototoxic burden damages cellular components vital for performance of apoptosis and the cells disintegrate via a necrotic process (*Lavie G, et al. (1999), British Journal of Cancer 79: 423–432).* In addition, HYP has also been documented to cause cell death via autophagic mechanisms (*Rubio N, et al. (2012), Autophagy 8, 1312–1324*). These authors found that following an ROS insult on the endoplasmic reticulum (ER), lipid hydroperoxides or their metabolites are produced which stimulate an ER-selective autophagy (reticulophagy). This oxidative damage is rapidly conveyed to the mitochondrial phospholipid which mediates a step of mitochondrial removal from affected cells and eventually also apoptosis.

HYP-PDT-mediated tumor damage to TCC tumors transplanted into rats has been observed with partial positive results without damaging adjacent normal tissue (*Kamuhabwa AA, et al. (2003), Int. J. Cancer. 107, 460–467*). Similar studies have been conducted *in vivo* in a large number of different tissues by various groups. In some, the selective HYP accumulation within tumor cells combined with the fluorescent properties of HYP, have been utilized to localize the tumor in the animal. For example, when human disseminated alveolar rhabdomyosarcoma cells were inoculated into SCID mice the fluorescent properties of HYP were used to localize the tumor and light irradiation was subsequently used to induce phototoxic tumor cell death. Tumor cell apoptosis has been demonstrated in these studies (*Urla C, et al. (2015), Surg. Endosc. 29, 1105–1114*). In mice bearing human epidermoid carcinoma A431 cell xenografts implanted intraperitoneally, complete inhibition of tumor growth has been achieved with 2.5 mg/kg HYP and light fluency of 180 J/cm^2 of cold white light (*Vandenbogaerde AL, (1996*), *Anticancer Res. 16, 1619–1625*). With murine fibrosarcoma G:5:1:13 cells inoculated into C3H/DiSn mice, complete response to PDT has been observed in 44.4% of the animals in which HYP was administered intraperitoneally and in 33.3% of animals in which HYP was administered into the tumor. Complete remission was also reported to occur in HYP-PDT treated lesions up to 3 mm in height. The treatment was reported to increase animal survival significantly (*Čavarga I, et al. (2001), Phytomedicine 8, 325–330*).

The effects of HYP photoactivated in both primary cell cultures and cell lines (squamous Kyse-140 and adenocarcinoma OE-33) from human esophageal cancer, have also been studied using an incoherent white light source. The esophageal cancer cells were preincubated with HYP doses ranging between 10nM - 1μM for durations of 4-24 hrs and subsequently irradiated with 30 J/cm^2 of white light. The authors observed strong phototoxic effects that induced apoptosis in a HYP dose-dependent manner with an IC(50) of approximately 30nM of HYP in both squamous and adenocarcinoma cells (*Hopfner M, et al. (2003), Int. J Colorectal Dis. 18: 239-247*).

3.1. Antimetastatic activities of Hypericin

Another most critical aspect of tumorigenesis is formation of metastases, the major cause of treatment failures and patient death. We have achieved selective focus on metastases by inoculating breast carcinoma DA3 and squamous cell carcinoma (SCC) SQ2 cells intradermally to mice to form primary tumors and resected these primary tumors when DA3 tumors reached 7-8 mm in diameter and SQ2 tumors 10-12 mm. At these stages micrometastases already existed in the lungs. The metastases bearing mice received 3-5 treatment schedules of HYP, 5 mg/kg at 5 day intervals intraperitoneally. The animals were kept under covered cages to minimize exposure to ambient light (< 0.03 mW/cm^2) and animal survival was monitored. Long-term animal survival increased in DA3 tumor-excised groups from 15.6% in untreated controls to 34.5% following treatments with HYP. In mice bearing SQ2 tumor metastases, treatment with HYP increased animal survival from 17.7% in controls to 46.1% in the HYP treated group (*Blank M, et al. (2004), Int. J.*

Cancer: 111, 596–603). These observations revealed that treatments with HYP were associated with marked anti metastatic effects the magnitudes of which were found to be directly related to the number of HYP dose regimens which were administered. After 160 days of follow-up, percent animal survival increased from 16 $\pm$ 7% (survival $\pm$ S.D.) in the resected animal controls not treated with HYP to 25 $\pm$ 7% in the group which received 4 HYP treatment regimens and to 35 $\pm$ 7% in the group which received 6 HYP regimens. Thus, animal treatment with HYP under conditions which selectively monitor formation of metastases show that the compound displays additional beneficial effects which may potentially be further improved by increasing the number of treatment regimens and dosages of HYP.

3.2. Anticancer activities of Hypericin in the dark

In addition to HYP-PDT, we have identified existence of anticancer activities of HYP in the dark. We analyzed effects of HYP applied at concentrations of 0.2, 1, 3, 10 & 20 μM on murine $DA3^{Hi}$ (highly metastatic) breast carcinoma cells and SQ2, squamous carcinoma cells cultured under strict darkness from the time of HYP administration compared to cultures exposed to polychromatic fluorescent light doses of 7.2 J/cm^2. Both groups were cultured for 24 and 72 hrs, and cell viabilities were monitored (using MTT assay). While cell death was widespread in the light-irradiated cultures, significant inhibition of tumor cell growth was also noted in the cultures which were maintained in the dark for 24 hrs, indicating that the effect was primarily cytostatic. However, when cultivation with HYP was extended to 72 hrs, cytocidal effects were also observed in cultures exposed to HYP at doses of 10 & 20 μM in the dark (ambient light kept < 0.03 mW/cm^2) (*Blank M, et al. (2001), Photochem Photobiol, 74: 120–125*). ^{3}H-thymidine incorporation into newly synthesized DNA following 72 hrs of cell treatment with HYP in the dark revealed an approximately 50% reduction in overall ^{3}H-thymidine incorporation relative to untreated controls.

The effects of systemic HYP administrations on survival of tumor-bearing animals have also been examined in the SCC model *in vivo*. Male BALB/c mice were inoculated with 5 x 10^5 SQ2 cells/mouse in three groups (10 mice each). Each group received a weekly dose of 5 mg/kg and 10 mg/kg (equivalent to 100 and 200 μg/mouse, respectively) intraperitoneally, and the third served as untreated controls. HYP treatments were begun when the tumors reached 4–6 mm in diameter (day 30) and continued for 9 weeks. Mean survival time was extended significantly from 115.7 $\pm$ 27.5 days in the untreated control group, to 152.1 $\pm$ 39.8 days ($P \leq 0.029$) in the 5 mg/kg group and to 166.5 $\pm$ 58.1 days in the 10 mg/kg ($P \leq 0.022$) group. (*Blank M, et al. Photochem Photobiol, 74: 120–125, 2001*).

The findings in the breast and squamous cell carcinoma experiments show that HYP possesses anticancer activities against these highly invasive tumors in animal models in the absence of any light irradiation. Thus, limiting the therapeutic scope of HYP to light dependent PDT indications understates the full therapeutic potential of this molecule. It is important to note, though that not all tumor types undergo growth arrest in response to treatment with HYP in the dark. For example, murine B16

melanoma cells appear to be more resistant to HYP therapy and 5–10-fold increases in HYP concentrations were required in order to affect B16 cell replication (*Blank M, et al. (2001), Photochem Photobiol, 74: 120–125*).

4. Clinical trials with Hypericin-PDT

Clinical trials with HYP-PDT followed more cautious and conservative approaches considering the limited skin penetration of light. They were therefore, primarily confined to treating superficial lesions of tumors such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) with HYP-PDT. (*Alecu M, et al. (1998), Anticancer Res. 18: 4651–4654*).

Patients with SCC were given 40-100 micrograms HYP intralesionally, 3-5 times/week for 2-4 weeks and patients with BCC were given 40-200 micrograms HYP intralesionally, 3-5 times/week for 2-6 weeks. After the injections, the affected areas were irradiated with visible light. The authors concluded that clinical remissions could be expected after 6-8 weeks and that there were no adverse toxicity effects to the surrounding healthy tissue (*Alecu M, et al. (1998), Anticancer Res. 18: 4651–4654*).

Another attempt at utilizing HYP-PDT to improve *in vivo* tumor growth control has been performed on nasopharyngeal carcinoma (NPC). However, since PDT has been found to increase cyclooxygenase-2 levels, the HYP-PDT treatment was combined with the COX-2 inhibitor, Celebrex (CX). The effect of an initial CX dose at 6- and 24-h post- HYP-PDT was investigated simultaneously. Bulk tumor ablation at 48-h post-PDT was observed. However, after 24-28 days tumor regrowth has been observed. In a combination treatment in which the 1st CX dose was administered 6-h post-PDT the highest level of tumor growth inhibition observed was (64.29%, $P<0.05$). However, tumors in which the initial CX dose was administered 24-h after the PDT no tumor control was observed. Co-suppression of COX-2, HIF-1α and VEGF A genes were noted in HYP-treated tumors compared to tumor controls. (*Yee KK, et al. (2005), Int J Mol Med. 16: 993-1002*). These experiments emphasize the need for a conservative approach and caution before using HYP-PDT in the therapy of invasive malignancies.

The most advanced clinical utilization of HYP-PDT has been in a placebo-controlled clinical study performed on 12 patients with early stage cutaneous T cell lymphoma. The objectives of the study were to determine if topical PDT with HYP is an effective, safe and well tolerated therapy for this disease. HYP was applied topically onto the lesions twice/week for 6 weeks and light was irradiated after 24 hrs at 8-20 J/cm^2. The study found that 58.3% of the patients responded to the treatment and HYP-PDT was well tolerated by the patients. (*Rook AH, et al. (2010), J Am. Acad. Dermatol. 63, 984–990*). This study protocol has currently advanced to Phase III.

4.1. Clinical trials of Hypericin in the dark

One clinical trial that aimed to evaluate the anticancer effects of orally administered, daily doses of synthetic HYP has been performed in recurrent malignant glioma patients (anaplastic astrocytoma and glioblastoma) with recurrent progressive

disease who had previously received radio and chemotherapies. The study was based on the presumption that HYP is a potent protein kinase C inhibitor (*Couldwell WT, et al. (1992), Neurosurgery 31: 717-724*) and that modulation of protein kinase C in human glioma cell lines produced growth inhibition in these cell lines (*Couldwell WT et al. (1990), J Neurosurg. 73: 594-600*).

The three month study in GBM was conducted as an open-label, sequential dose escalation/de-escalation tolerance study which had as end points achievement of reduction in tumor volume and prolongation of survival. HYP produced stabilization or a slight decrease in tumor volume (<50% reduced tumor size) (stable disease) in 7 of 42 patients (17%) and a tumor reduction >50% (partial response) in 2 other patients (4%), an overall response rate of 22%. Seventeen patients (40%) survived the entire 3 month study and the median patient survival was 26 weeks (6 months). (*Couldwell WT, et al. (2011), Cancer 117: 4905-4915*). Twelve patients continued on oral HYP therapy beyond the 3 months of the study on a compassionate basis. Eight of 42 patients survived beyond 6 months (mean survival, 20.8±11.35 months). One patient survived beyond 36 months. Further analyses indicated that the mean progression-free survival of all patients in the intent-to-treat group was 20 weeks. These findings were found to be within the efficacy range of all other conventional therapies used to treat GBM (*Couldwell WT, et al. (2011), Cancer 117: 4905-4915).*

5. Mechanisms of the anticancer activities of Hypericin

In apoptosis-resistant solid tumor cells such as murine breast carcinoma, HYP was found to elicit mitotic cell death (MCD) forming the characteristic giant multimicronucleated polykaryon cells (*Blank M, et al. (2003), Cancer Res. 63: 8241-8247*).

We conducted studies aimed at understanding the underlying mechanism for the anticancer activities of HYP and for the occurrence of MCD as the route of cell death induction. We found in murine breast cancer DA3 cells that treatment with HYP (in the dark) caused marked reductions and rapid elimination of the heat shock protein Hsp90 from these cells. HYP was found to bind preferentially to Hsp90 (Fig. 2) and to induce an oxidation which as a consequence, activated the ubiquitin pathway, subjecting Hsp90 to forced, accelerated polyubiquitinylation, functionally inactivating Hsp90. Hsp90 was rapidly degraded and eliminated from the breast cancer cells in a proteasome independent manner. (*Blank M, et al. (2003), Cancer Res. 63: 8241-8247).*

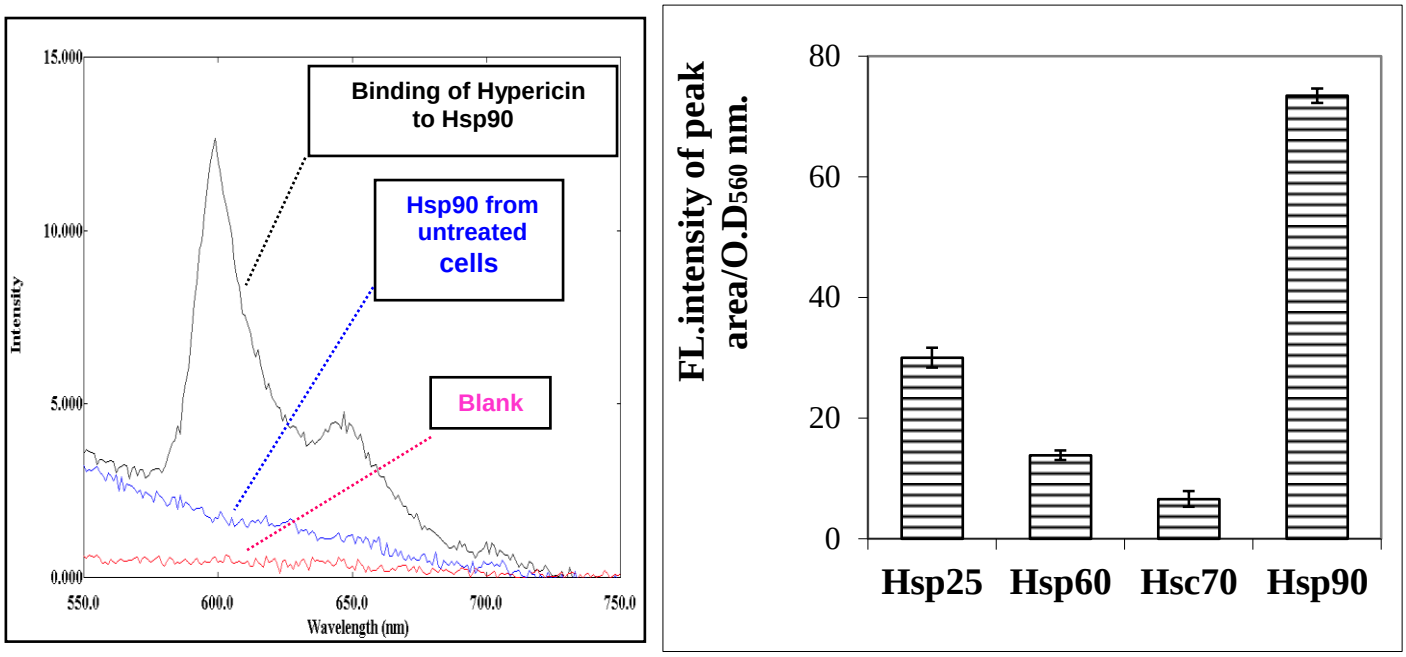

Figure 2. Preferential binding of Hypericin to Hsp90: Immunoprecipitation with antibodies to several heat shock proteins followed by Laser-Induced Flourescence Analysis

This scenario has far reaching consequences because the Hsp90 chaperone, which is at the hub of homeostasis (*Taipale M, et al. (2010), Nature Rev Molecular Cell Biol. 11, 515–528, Review*), normally binds to and forms complexes with a very large and heterogeneous group of Hsp90 client proteins. Hsp90 assists in folding and remains bound to these client proteins, thereby continuing to protect their intracellular stability and functional activities. These client proteins include various ser/thr and tyrosine kinases, steroid hormone receptors, mutated p53, co-chaperone p23 (*Felts et al. Cell Stress Chaperones. (2003), 8: 108–113*), the constitutively expressed cognate hsc70, the Hsp90-organizing protein HOP (also known as p60), which stimulates recruitment of Hsp90 (*Smith DF, et al. (1993), Mol Cell Biol. 13: 869-876*), the Hsp40–Hsp70 chaperone system (*Bogumil D, et al. (2014), Mol Biol Evol. 31: 410–418*), cyclin D-associated Cdk4 (*Helmbrecht, K, Zeise E, Rensing L. (2000) Cell Prolif. 33: 341–365*), Cdk6, HDAC6, wild-type polo-like kinase (Plk), Raf-1, the hypoxia response proteins - hypoxia-inducible factors 1α HIF1α, HIF2α, HIF3α, (*Minet E, et al. (1999), FEBS Lett. 460: 251–256*), ERBB2, HCK, EGFR, BRAF STAT3, mutant p53 and many others.

As a consequence, signaling pathways such as the Ras/Raf pathway are inhibited, their downstream extracellular signal-regulated kinase 1/2 (ERK 1/2) are prevented from being phosphorylated and

activated, bringing tumor cell proliferation to a halt (*Blank M, et al. (2003), Cancer Res. 63: 8241-8247*). Inhibition of the Ras/Raf/Erk pathway is also highly important in the fight against glioblastoma. This most aggressive type of tumor develops due to deregulation of cell proliferation pathways, primarily the Ras/Erk pathway which becomes constitutively activated. The tumor suppressor PTEN gene which dephosphorylates phosphatidylinositol 3,4,5-trisphosphate becomes inactivated and consequently causes elevation of AKT in a majority of GBM tumors. Constitutive activation of AKT and the AKT/mTOR signaling pathway then occurs (*Haas-Kogan D, et al. (1998), Current Biology 8:1195–1198*). Degradation of Plk involved with CDK/cyclin complexes in mitotic spindle formation during cell cycle M-phase, prevents cell crossing into the M-phase, and completion of the cell cycle.

The destabilization and enhanced turnover of Hsp90 client proteins was found to be associated with marked declines in the cellular contents of additional cell cycling regulators: cellular levels of cyclin A, cyclin B1, and cyclin H (the regulatory subunit of cyclin activating kinase) (*Martinez AM, et al. (1997), EMBO J. 16: 343–354; Kato JY, et al. (1994), Mol. Cell. Biol. 14: 2713–2721*) diminish in HYP dose-related manners. The G1 checkpoint inhibitor $p27^{kip1}$ cellular level also decreases and is associated with elevated cyclin E, predominantly in the nuclei. This latter combination, particularly the reduced cell contents of $p27^{kip1}$, cyclin A and cyclin B1 result in lack of their activities, leading to diminishing residual G1 checkpoint activity and cell progression to S phase occurs in a dysregulated manner after treatment with HYP. Orderly completion of DNA replication after S phase is disrupted and in the absence of functional G2/M checkpoint activity the cells go into premature mitosis. A cycle of repeated DNA replications and karyokinesis (replication of the nuclei) then occurs in the absence of cytokinesis (separation of the cytosol), leading to formation of multiple micronuclei. The resulting process is an uneven mitosis termed "mitotic catastrophe" which is characterized by formation of polykaryonic giant cells, the hallmark of "mitotic cell death" (MCD) (*Torres K, et al. (1998), Cancer Res. 58: 3620-3626*). MCD has indeed been found to occur following exposure of DA3 murine breast carcinoma cells, SQ2 murine squamous carcinoma cells and also B-16 melanoma cells to HYP in the dark (*Blank M, et al. (2003), Cancer Res. 63: 8241-8247*).

6. Antiangiogenic effects of Hypericin

In addition to anti-cancer activities which directly target the tumor cells, HYP is also a potent anti-angiogenic agent, an activity which also occurs in the dark. In several *in vitro* studies HYP was demonstrated to inhibit major steps in the angiogenic path such as endothelial cell proliferation, cell migration, invasion and tubule formation in matrigels (*Martínez-Poveda B, et al. (2005), Eur. J. Pharmacol. 516, 97–103*).

In vivo, the extensive angiogenic responses induced in the iris of rat eyes by intracameral administration of FGF-2 and in the cornea by superficial FGF-2 administration to the anterior eye chamber, were effectively inhibited with HYP. A

minimum of 4 intraperitoneal HYP doses (2 mg/kg) given at 48 hr intervals were sufficient to abrogate angiogenesis and maximal inhibition occurred when HYP administrations were begun 48 hrs prior to FGF-2 inoculation (*Lavie G, et al. (2005), Angiogenesis 8: 35-42*). Our studies further showed that exposure of ARPE-19 human retinal pigment epithelial cells or EA.hy926 vascular endothelial hybridoma cells to HYP in cultures (in the dark), caused inhibition of ERK1 & 2, MAP kinase phosphorylation. HYP treatment inhibited this signaling pathway which is vital for angiogenesis and which functions via the phosphorylating activation of the ERK1/2 MAP kinases. Furthermore, the effects of HYP on the VEGF-induced destabilization of existing blood vessels to form sprouting sites for newly forming microvasculature, have also been analyzed in human microvascular endothelial cells (HUMVECs) activated with VEGF in cultures. Activity profiles of matrix metalloproteases which mediate this microangiogenic process, by the cell membrane associated (MT1-MMP) (*Seiki M, et al. (2003), Cancer Sci 94: 569–574*), as well as by the secreted MMP2 and MMP9 (*Moses MA. (1997), Stem Cells 15: 180–189; Egeblad M, Werb Z. (2002), Nat Rev Cancer 2: 161–174*) were found to be modified by HYP in gelatin zymographies (*Lavie G, et al (2005), Angiogenesis 8: 35-42*).

Another intriguing effect of the HYP-mediated Hsp90 ubiquitinylation is the disruption of the tightly regulated cellular response to hypoxia. HIF-1α (or HIF-2α), the key regulator(s) of the response to hypoxia, are produced constitutively by most cells in organisms. Under hypoxic conditions HIF-1α binds to Hsp90 which helps direct HIF-1α into the cell nucleus where it associates with HIF-1β to form the active hypoxia-inducible factors HIF1 (or HIF-2). These active transcription factors (*Minet E, et al. (1999), FEBS Lett 460: 251–256*) then bind to the hypoxia response elements (HRE) on the promoters of angiogenic genes as VEGF, PDGF (platelet-derived growth factor) and other angiogenesis related genes, promoting their transcription and translation. VEGF (VEGF-A) which is synthesized stimulates increased expression of its primary receptor VEGFR2, forming a VEGF-VEGFR2 complex. This complex requires association with hsp90 to activate the downstream signaling that initiates the cascade triggering an angiogenic reponse and formation of new blood vessels (*Falkman J (1995), Nat Med 1: 27–31*). In cancer, neoangiogenesis constitutes a detrimental reaction, facilitating metastatic growth and spread. However, normally under normoxia, a conserved proline residue on HIF-1α is hydroxylated by proline hydroxylases, via an oxygen-dependent modification (*Min JH, et al. (2002), Science. 296: 1886–1889*). The proline hydroxylated HIF-1α triggers Von-Hippel Lindau tumor suppressor gene (pVHL) to form a complex of HIF-1α with pVHL, elongin B, elongin C, and cullin-2, a complex possessing E3 ubiquitin ligase activity (*Ohh M, et al. (1999), Journal of Clinic. Invest. 104: 1583–1591*). This complex can bind to and ubiquitinate HIF-1α directing it to rapid degradation in a proteasome dependent manner *(Maxwell PH et al. (1999), Nature 399: 271–275*).

This HIF-1α degradation constitutes a regulatory process which prevents occurrence of the response to hypoxia and synthesis of VEGF under normoxic

conditions (*Maxwell PH, et al. (1999), Nature 399: 271–275*). HYP, by destabilizing and degrading Hsp90, prevents HIF-α proteins from translocating to the cell nucleus, abrogating VEGF production and the hypoxia-induced angiogenic process in cancer cells is inhibited from occurring (*Barliya T, et al. (2011), PLoS ONE 6(9): e22849*). In the absence of angiogenesis the dissemination of metastases is also prevented (*Blank M, et al. (2004), Int. J. Cancer 111: 596–603*). Thus, the induction of forced Hsp90 polyubiquitinylation by HYP in cancer cells, which occurs in a proteasome independent manner (destabilization of Hsp90 client proteins is unaffected by the MG132 proteasome inhibitor), ties together the triggering of a direct HYP effect on the tumor cells, causing cell death via MCD with inhibition of a neoangiogenic response. Here, as well, HYP, by inducing a process of HIF-1α ubiquitinylation destabilizes and rapidly degrades HIF-1α (or HIF-2α) preventing HIF-1α from entering the cell nucleus. This process occurs independently of the proteasome and is an outcome of the direct HYP-induced forced Hsp90 ubiquitinylation and elimination. In summary, HYP, by preventing HIF-α proteins from translocating to the cell nucleus and inducing VEGF and PDGF production inhibits hypoxia-induced neoangiogensis from occurring in cancer cells (*Barliya T, et al. (2011), PLoS ONE 6(9): e22849. doi:10.1371/journal.pone.0022849*).

7. Induction of tumor cell differentiation by Hypericin

In a clinical trial performed by WT Couldwell and his colleagues in recurrent, progressive GBM and grade III anaplastic astrocytoma (AA) patients, the effects of orally administered HYP were evaluated on GBM tumor growth and patient survival. Stable disease has primarily been the prevalent observation in the HYP-responsive patient population. The tumors did not disappear yet they ceased growing and yielded a median patient survival time of 26 weeks (6 months) (*Couldwell WT, et al. (2011), Cancer. 117: 4905-4915).* Although tumor entrance into extended dormancy periods is a known phenomenon, the organized drug-dependent timing in which cytostasis had occurred following HYP therapy was more suggestive that tumor cell differentiation may have occurred.

Despite the difficulties to achieve tumor cell differentiation and cessation of cell proliferation, we found that GBM cell treatment with HYP in culture (also under dark conditions) was able to overcome the block in cell differentiation which characterizes various neoplastic malignancies and induce GBM tumor cell differentiation (*Dror N, et al. (2013), PloS One, 8(9):e73625*). Exposure of U251-MG and T98G human glioblastoma cell lines to HYP induced de novo expression of the neuronal differentiation antigens β III tubulin and NeuN. In U87-MG glioblastoma cells HYP induced expression of the glial cell antigen glial fibrillary acidic protein (GFAP) indicating an overall induction of GBM tumor cell differentiation to neuro-glial cells in the three cell lines.

In normal cells, expression of differentiation features is tightly linked to cell exit from replication cycles. Chromatin landscape changes controlling expression of differentiation related genes are linked to those controlling proliferation checkpoint genes, generating complete post-mitotic

differentiated cells. This linkage apparently, cannot be taken for granted in tumor cell differentiation.

HYP treatment of U87-MG and T98G cells did, indeed, result in thymidine incorporation shutdown (*Dror N, et al. (2013), PloS One, 8(9):e73625*), however, replication shutdown seemed less robust in HYP-induced U251-MG cell differentiation, albeit strong de-novo expression of β III tubulin and NeuN differentiation antigens. We were able to firm U251-MG cell proliferation arrest by combining HYP with HDACi as Trichostatin A or valproic acid treatment in this cell line. Nevertheless, these findings point to potential therapeutic flaws which may occur in incorporating epigenetic modulators such as HYP (or others) to tumor cell differentiation therapy in GBM.

8. Effects of Hypericin on epigenetic codes of glioblastoma cells

Because HYP induces forced polyubiquitinylation and rapid inactivation of Hsp90, the involvement of this chaperone in epigenetic modifications and the ability of HYP to inhibit transcription of HDAC genes prompted us to examine whether HYP can also affect cellular epigenetic and chromatin remodeling.

For differentiation to take place, relaxation of the abnormally compact organization of tumor cell chromatin must first be achieved. Compact heterochromatin is highly suppressive for gene expression and patterns of cancer cell gene expression are modulated via adjustments in epigenetic transcriptional codes. The best-known epigenetic modulator in gliomas is DNA methylation. Hypermethylation of gene promoters can silence genes which regulate the cell cycle, interfere with cell-to-cell interactions, diminish apoptosis, modulate DNA repair and promote neoangiogenesis. (*Parsons DW. et al. (2008), Science 321: 1807–1812).* Expression levels of the DNA methyl transferases - DNMT1 and DNMT3b are also abnormally elevated in GBM cells (*Kanai Y, et al. (2007) Carcinogenesis 28: 2434–2442; Fanelli M, et al. (2008), Oncogene 27: 358–365; Kreth S, et al. (2011), PLoS One 18:6(2): e17156*).

Cancer cell transcriptomes can also be modified by histone methylation, the patterns of which also affect the shape of cellular epigenetic codes. Histone methylation occurs via histone methyltransferases and regulates chromatin dynamics (*Sims RJ 3rd. (2003), Trends Genet. 19: 629-639*). One enzyme complex which performs histone methylation is the Polycomb repressive complex-2 (PRC2). PRC2 is the methyltransferase which tri-methylates histone H3 lysine-27 to H3-K27-3me and is also implicated in carcinogenesis (*Cao R, et al. (2004), Curr Opin Genet Dev 14: 155–164*). The catalytic subunit of the PRC2 complex – EZH2, is found to be abnormally elevated in numerous tumor types including GBM and the gene locus of EZH2 is specifically amplified in several primary tumors. In fact, the EZH2 and SUZ12 components of the PRC2 complex, have always been found to be abnormally elevated in tumors, including GBM tumors (*Bracken AP, et al. (2003), Embo J. 22:5323–5335*). The elevated EZH2 measured levels were found to correlate with advanced disease stage and poor prognosis, with the highest levels significantly correlating with tumor grade in both adult and pediatric brain cancers (*Crea F, et al.*

(2010), Mol Cancer 9: 265). To generate aberrant epigenetic machineries which form dysregulated gene promoter methylation patterns and produce transcriptionally repressed heterochromatin, EZH2 must form physical interactions and functional links with HDACs (*Van Der Vlag J, et al. (1999), Nat Genet 23: 474–478*) and with all three DNA methyl transferases (DNMTs) (*Vire E, et al. (2006), Nature 439: 871–874*).

We found that treatment of U87-MG, T98G and U251-MG human GBM cell lines with HYP caused dramatic reductions in the intracellular contents of the PRC2 complex catalytic subunit EZH2, as well as in the cell contents of DNMT1 and HDAC1. These three enzymes interact with each other forming links with other DNMTs. They engage in formation of highly regulated functional complexes which are abnormal in cancers due to high expressions of HDACs and EZH2 (*Simon JA, et al. (2008), Mutation Res 647: 21–29*) and were, thus associated with the HYP-mediated marked decreases in histone H3, lys27 trimethlation (H3K27-3me). HYP treatment appears to have corrected some of these aberrations possibly by downregulating HDACs, DNMT1 and EZH2 (*Dror N, et al. (2013), PloS One, 8(9):e73625*). The aberrant expression profiles of DNMTs found in GBM cells such as up-regulation of DNMT1 and down-regulation of the de novo DNMT3a (*Fanelli M, et al. (2008), Oncogene 27: 358–365*) were also modified by HYP and in U87-MG cells DNMT3b expression was down-regulated in a HYP dose dependent manner (*Dror N, et al. (2013), PloS One, 8(9):e73625*). The DNMT profiles generated by the cell treatment with HYP were totally incompatible with the abnormal profiles of these enzymes in GBM cells. Indeed HYP-induces increases in global DNA methylation, which may help stabilize the GBM cell genome and is likely to elicit functional DNMT activity changes, bearing closer similarities to those of normal cells.

Another highly relevant participant in the shaping of epigenetic codes and chromatin conformation is histone lysine acetylation. Mediated by histone acetyl transferases, histone acetylation, which also relaxes chromatin structure enables better access of transcription factors to DNA promoter sites and is associated with transcriptional activation (*Fry CJ et al. (2001), Curr Biol 11:R185–R197; Fry CJ, et al. (2002), Science 295: 1847–1848*; *Workman JL, et al (1998), Annu Rev Biochem. 67: 545–579*). Histone acetylation also contributes to inhibition of GBM tumor cell proliferation *in vitro* (*Kamitani H, et al. (2002), Neuro-Oncology 4: 95-101*). Histone acetylation is therefore, a diagnostic feature of transcriptionally active genes (*Kang S-K, et al. (2006), Stem Cells and Develop. 15: 165-174*).

Hypoacetylation of histones H3 and H4, on the other hand, increases chromatin compaction, preventing transcription of many genes including cell cycle checkpoints, DNA repair genes and differentiation-related genes. The abnormal malignancy-promoting epigenetic codes include hypoacetylation of histones H3 and H4, which characterize various cancer forms including GBM (*Cadieux B, et al. (2006) Cancer Res 66: 8469–8476*). These hypoacetylations increase chromatin compaction, reduce gene transcription, abrogate cell replication arrest, inhibit cell differentiation and prevent apoptosis. (*Moss TJ, et al. (2007), Mutat Res 618: 163–174;*

Sparmann A, et al. (2006), Nat Rev Cancer 6: 846–856).

Histone hypoacetylation results from excessive activity of HDACs and occurs in various malignancies including GBM *(Timmermann S, et al. (2001), Cell Mol Life Sci 58: 728–736).* GBM cells regularly display strong nuclear expression of the Class I HDACs - HDAC1 and HDAC2, contributing to chromatin compaction. Furthermore, HDAC1 and HDAC2 expressions have been found to increase during tumor recurrence and tumor progression, respectively (*Campos B, et al. (2011), Cancer Sci. 2011 102: 387-392*); class II and class IV HDACs appear to cause decreased mRNA expression in GBM tumors compared to low-grade astrocytomas and normal brain samples. Since epigenetic aberrations play critical roles in formation of neoplasia-promoting platforms (*Timmermann S, et al. (2001), Cell Mol Life Sci 58: 728–736),* these aberrations, in fact, have become targets for anticancer therapy (*Mack GS. (2006). J Natl. Cancer Inst. 98: 1443–1444*). The aim has been to relax the compacted cancer cell chromatin, which prevents access of transcription factors to silenced promoters including those of cell cycle checkpoints and differentiation-related genes (*Bernstein BE, et al. (2007), Cell 128: 669–681*). Such goals appeared to have become achievable through increasing histone acetylation levels by using small molecule histone deacetylase inhibitors (HDACi). HDACi have been found to overcome blocks in tumor cell differentiation, reactivate apoptosis and alter angiogenesis (*Bolden JE, et al. (2006), Nat Rev Drug Discov. 5: 769–784; Benítez JA, et al. (2008), Neuroscience 156, 4, 911-920; Marks PA et al. (2000), J Natl Cancer Inst. 92: 1210-1216; Rosato RR, et al. (2003), Cancer Res. 63, 3637-3645*). Nevertheless, consistent clinical benefits have, thus far been confined primarily to subtypes of hematologic malignancies and could not be achieved consistently in various types of solid tumors (*Kreth S, et al. (2011), PLoS One 18:6(2): e17156*).

Since HDACi are capable of inducing tumor cell differentiation we examined whether HYP is also an HDACi, however measurements of HDAC catalytic activities following cell exposures to HYP for 72 hrs did not show the high deacetylation activity inhibition expected from HDAC inhibitors (*Dror N, et al. (2013), PloS One, 8(9):e73625*). They did show, however, reductions in deacetylating activities in the ranges of 59.6%$\pm$10.5% ($p \leq 0.047$) in U87-MG cells and 53.6%$\pm$14.9% ($p < 0.035$) in U251-MG cells. These values do not reflect enzymatic inhibition and suggest that HYP affects cellular HDACs differently. We, therefore, examined HYP effects on the actual expression levels of HDACs in these cell lines. HDAC1, HDAC2 and HDAC3 protein expression evaluations showed HYP dose-dependent reductions in HDAC1, HDAC2 and HDAC3 cell contents in all three cell lines, although HDAC3 decreases were less consistent in U251-MG and T98G cells. HYP effects on transcription of the three HDAC genes by qRT-PCR analyses, performed on RNA from these cells following 48 hr exposures to HYP, revealed 32–55% decreases in HDAC1 mRNA levels in U87-MG cells, in response to 10–50μM HYP (significant in the 10–30μM range). Reductions of 10–50% occurred in HDAC2 cellular contents (significant with 40μM HYP). T98G cell HDAC1 mRNA decreases were mild (5–25%), yet HDAC2 decreases

ranged between 4–40% (both values significant with 20μM HYP), and HDAC3 decreased 16–41% with 10–50μM HYP, respectively (significant in 20–40μM dose range). In U251-MG cells HDAC1 mRNA levels showed trends toward 22–25% decreases in expression, HDAC2 mRNA declined 27–32% (significant with 20 and 40μM HYP). HDAC3 mRNA levels showed trends towards 40–50% reduced contents. HDAC2 levels were reduced by 10–50% (significant with 40μM HYP) (*Dror N, et al. (2013). PloS One, 8(9):e73625*). These findings point to HYP-mediated reductions in transcription of the three HDAC genes and suggest HYP-mediated epigenetic modulatory effects on promoter regions of HDACs in these GBM tumor cells. We have also shown that by downregulating expressions of HDACs HYP indirectly causes increased histone acetylations and thereby reduced abnormal DNMT profiles modifying the tumorigenic codes. (*Dror N, et al. (2013), PloS One, 8(9):e73625*). Also modified by HYP are the aberrations in epigenetic platforms and dysregulated oncogenic DNA methylation patterns which promote genomic instability and facilitate GBM oncogenesis (*Esteller M (2008), Epigenetics N Engl J Med 358: 1148–1159; Ballestar E, et al. (2003). EMBO J., 22:6335-6345).* Thus, HYP exerts epigenetic anti-GBM activities which induce post-mitotic GBM tumor cell differentiation. Although these studies were performed on cell cultures, we believe they may explain the clinical findings obtained in the Phase I/II clinical trial conducted in recurrent progressive GBM patients who failed other therapies (*Couldwell WT, et al. (2011), Cancer 117: 4905-4915*). In this study 35 GBM and 7 anaplastic astrocytoma (AA) patients with recurrent, progressive disease who failed other therapies were enrolled to a three month trial which evaluated changes in tumor size and patient survival in response to daily orally administered HYP. Forty percent (17 of 42) of the patients completed the three month trial. Forty one percent of the responder group (7 of 17 patients) achieved stable disease, defined as no definitive increase in tumor size as determined by MRI. Twelve percent (2 of 17 patients) showed a partial response defined as a greater than 50% reduction in tumor area. Patients who remained alive at the end of the three month study were continued on HYP on a compassionate basis. The median survival time was 6 months with 8 of the patients surviving beyond 6 months (3 survived for 11-12 months, two survived for over 36 months (*Couldwell WT, et al. (2011), Cancer 117: 4905-4915*). With the tumors remaining in place in most of the surviving patients, it was evident that the tumors in the HYP treated GBM patients have undergone dramatic functional and behavioral changes which prevented the continued growth of these tumors. We hypothesized that these tumors have been undergoing cell differentiation.

8.1. Possible role of Hsp90 in Hypericin-induced reshaping of cancer cell epigenetic codes

The Hsp90 chaperone has also been implicated in forming links between biochemical chaperone activities and epigenetic gene regulation in developing organisms (*Zhao R, et al. (2005). Cell 120: 715–727*). These links were discovered following extensive efforts to characterize and map all physical and genetic Hsp90 interactions in yeast. Physical Hsp90 interactions were found using genome-wide

two hybrid screens combined with large-scale affinity purification of Hsp90-containing protein complexes. Genetic interactions were uncovered using synthetic genetic array technology combined with a microarray-based screen of a set of ~4700 viable yeast gene deletion mutants, analyzed for hypersensitivity to the Hsp90 inhibitor geldanamycin. These analyses identified two novel Hsp90 cofactors, Tah1 and Pih1 which interact physically and functionally with the conserved AAA(+)-type DNA helicases Rvb1 and Rvb2. Both are key RuvB-like AAA+ DNA helicases linked to the Ino80 chromatin remodeling complex and to other core components of the SWR-C chromatin remodeling complex in yeast (*Zhao R, et al. (2005), Cell 120: 715–727; Jonsson ZO, et al.(2001), J. Biol Chem. 276, 16279-16288*). Similar Hsp90 involvement in chromatin conformation modifications have been found in mammals in which Hsp90 has been shown to increase the activity of the histone H3 lysine-4 methyltransferase SMYD3, which activates the chromatin of several target genes. Hsp90 has also been found to capacitate morphological evolution by masking epigenetic variation (*Ruden DM, et al. (2005), Human Molecular Genetics, Vol. 14, Review Issue 1, R149-R155*).

The Hsp90 molecule also undergoes acetylations which modify its activities. In leukemic cells this chaperone has been found to be deacetylated by HDAC6, itself an Hsp90 client protein (*Rao R, et al. (2008) Blood 112, 1886-1893*). A complex interplay exists between HDAC6 and Hsp90 in which Hsp90 is deacetylated by HDAC6 in the context (among others) of regulating autophagy by Hsp90 (*Reikvam H, et al. (2009), Curr. Cancer Drug Targets 9, 761-776*). The HYP-induced forced polyubiquitinylation which leads to functional Hsp90 inactivation and accelerated degradation (*Blank et al. (2003), Cancer Res. 63: 8241-8247*), inevitably also eliminates any biological activities mastered by the Hsp90-HDAC6 interplay or any of their implications.

9. Conclusions

Current anticancer trends contemplate replacing cytotoxic chemo and radiotherapies with molecular targeted therapy. Four approaches were developed: [1] synthesis of receptor tyrosine kinase inhibitors based on rational drug design such as imatinib mesylate used to treat CML, [2] development of tumor cell differentiation inducers as HDAC inhibitors with suberoylanilide hydroxamic acid SAHA already approved for use to treat CTCL, [3] ubiquitin-proteasome pathway modulators as the proteasome inhibitor Bortezomib used to treat multiple myeloma and [4] angiogenesis inhibitors as bevacizumab used in several recurrent & metastatic cancers.

The perylene quinone HYP is shown to touch upon all 4 targeted anti-cancer approaches. The potent photodynamic properties of HYP elicit a range of light-dependent tumoricidal activities. Yet, a relatively low redox potential endows HYP with electron accepting and donating properties, enabling it to act as both an oxidizing and a reducing agent. HYP can thus compete as an electron acceptor from bioenergized reduction/oxidation reactions, generating its excitation energy for biological activities from physiological redox reactions in the absence of light.

The combination of lipophilic properties, which attract HYP to cell

membranes with redox activities render HYP capable of acting as the only known exogenous reagent that can selectively target an intracellular regulatory protein - Hsp90 to undergo forced polyubiquitination and accelerated degradation and elimination. As a consequence, the plethora of Hsp90 client proteins are also destabilized and degraded. Several such Hsp90 client proteins are signaling mediators of cell replication pathways which become deficient, bringing replication of several cancer cell types to a halt. Another Hsp90 client protein – HIF-1α, is also destabilized and degraded. HYP has been shown to accelerate HIF-1α degradation even under hypoxic conditions, in which HIF-1α is normally stabilized, downregulating VEGF expression profiles and VEGFR2 transcription and protein expression. HIF-1α normally activates VEGF synthesis inducing angiogenesis and its ablation blocks angiogenesis. Consequently, HYP is a robust angiogenesis inhibitor in matrigel assays and in rat eye pocket models, affecting multiple sites in the angiogenic cascade. The inhibition of neoangiogenesis renders HYP an effective inhibitor of tumor metastasis.

HYP also displays a unique pattern of epigenetic modulations. GBM cell treatment with HYP effectively downregulates class 1 HDAC1, HDAC2 and HDAC3, and class 2b HDAC6 and HDAC10, resulting in histones H3 & H4 becoming highly acetylated.

The downregulation of HDAC1 is associated with strongly reduced expression of DNMT1 and EZH2 with which HDAC1 forms physical and functional complexes, depleting the functions of the PRC2 histone methylating complex. Together with the HYP-generated deficiencies in HDACs this new platform induces post mitotic GBM tumor cell differentiation in human GBM and murine breast carcinoma cells. Tumor cell differentiation is evident by increased neuro-glial differentiation antigen expression, associated, in the majority of cases, with DNA replication shutdown. HYP can inhibit the growth of highly metastatic murine breast adenocarcinoma and squamous cell carcinoma tumors in culture and can interfere with the growth of these tumors in mice, reducing tumor size and prolonging animal survival in complete absence of light.

The multi-targeted activity of HYP translates into clinical anti-GBM tumor efficacy in the treatment of brain tumors. A multicenter phase I/II clinical trial conducted in 42 advanced recurrent patients with, grade IV glioblastoma and grade III anaplastic astrocytoma who failed other therapies resulted in 20 week PFS and 26 week median survival with daily oral HYP as monotherapy. From the group of patients who completed the trial and put on a compassionate treatment arm, two patients survived > 34 months and three others survived for 11-12 months. Adverse effects were limited primarily to phototoxicity. A topical HYP 6 week phototherapy trial conducted in patients with early stage CTCL resulted in 58% positive response rates. Thus HYP is emerging as a potent, safe anticancer agent with antiangiogenic and anti-metastatic properties.

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