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Hypericin affects cancer side populations via competitive inhibition of BCRP

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ABSTRACT

Objective: Cancer stem-like cells (CSLCs) are considered a root of tumorigenicity and resistance. However, their identification remains challenging. The use of the side population (SP) assay as a credible marker of CSLCs remains controversial. The SP assay relies on the elevated activity of ABC transporters that, in turn, can be modulated by hypericin (HYP), a photosensitizer and bioactive compound of St. John's Wort (*Hypericum perforatum*), a popular over-the-counter antidepressant. Here we aimed to comprehensively characterize the SP phenotype of cancer cells and to determine the impact of HYP on these cells.

Methods: Flow cytometry and sorting-based assays were employed, including CD24-, CD44-, CD133-, and ALDH-positivity, clonogenicity, 3D-forming ability, ABC transporter expression and activity, and intracellular accumulation of HYP/Hoechst 33342. The tumorigenic ability of SP, nonSP, and HYP-treated cells was verified by xenotransplantation into immunodeficient mice.

Results: The SP phenotype was associated with elevated expression of several investigated transporters and more intensive growth in non-adherent conditions but not with higher clonogenicity, tumorigenicity or ALDH-positivity. Despite stimulated BCRP level and MRP1 activity, HYP reversibly decreased the SP proportion, presumably via competitive inhibition of BCRP. HYP-selected SP cells acquired additional traits of resistance and extensively eliminated HYP.

Conclusions: Our results suggest that SP is not an unequivocal CSLC-marker. However, SP could play an important role in modulating HYP-treatment and serve as a negative predictive tool for HYP-based therapies. Moreover, the use of supplements containing HYP by cancer patients should be carefully considered, due to its proposed effect on drug efflux and complex impact on tumor cells, which have not yet been sufficiently characterized.

1. Introduction

A concept of so-called cancer stem-like cells (CSLCs) is an attractive notion that helps to explain various aspects of tumor diseases, including tumorigenicity, resistance, and metastasis [1]. However, it seems that the multifaceted nature of cancer excludes the possibility of identifying a single universal marker, or a reasonable number of them, applicable in CSLC identification. Since the crucial features of CSLCs are self-renewal and lineage capacity, serial orthotopic xenotransplantations represent the gold standard for their identification [1]. However, *in vivo* experiments are expensive and time-consuming, and thus ineffective. Therefore, it was desirable to establish more robust *in vitro* methods for

Abbreviations: ABC transporters, ATP-binding cassette transporters; ALDH, aldehyde dehydrogenase; BCRP, breast carcinoma resistance protein; CDH1, cadherin 1 gene encoding E-cadherin; CSLCs, cancer stem-like cells; DEAB, N,N-diethylaminobenzaldehyde; EDTA, ethylenediaminetetraacetic acid; EMT, epithelial-mesenchymal transition; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FSC, forward scatter; H33342, Hoechst 33342; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hsp90, heat shock protein 90; HYP, hypericin; HYP-PDT, hypericin-mediated photodynamic therapy; LRP, lung resistance-related protein; MDR, multidrug resistance; MDR1, multidrug resistance protein 1; MR, medium replacement; MRP1, multidrug resistance-associated protein 1; NAD(P), nicotinamide-adenine dinucleotide phosphate; NSCLC, non-small cell lung carcinoma; P-gp, P-glycoprotein 1; PBS, phosphate buffered saline; PDD, photodynamic diagnosis; PDT, photodynamic therapy; PI, propidium iodide; PS, photosensitizer; RFU, relative fluorescence unit; SCID, severe combined immunodeficiency; SHO, SCID hairless outbred mouse; SJW, St. John's Wort; SP, side population; SSC, side scatter

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CSLC detection. 3-dimensional (3D) tumorosphere models represent a link between *in vivo* and *in vitro* assays and are employed in expansion of putative CSLCs. However, to select only the anchorage-independent CSLCs, tumorospheres must be derived from a single cell [2], which is typically laborious and low-throughput. On the other hand, SP analysis, which was originally described as a method for the identification of stem cells from mouse bone marrow [3], is more rapid and robust, and now widely utilized in CSLC-research. SP cells have increased ATP-binding cassette (ABC) transporters activity, which enables them to efflux DNA-binding dyes, such as Hoechst 33342 (H33342). As a result, SP emerges as a separate population with dimmer H33342 fluorescence shifted to the blue spectrum. However, it is well-known that the final size of the SP depends on many factors [4], making this method particularly sensitive. Although the SP phenotype is often linked to BCRP [5–7] and MDR1 (P-gp) [8] activities, it is not clear whether the H33342 efflux by CSLCs is mediated by only these or additional transporters and whether SP populations' identities mediated by different pumps overlap.

The measurement of aldehyde dehydrogenase (ALDH) activity has also been used to identify CSLCs [9–11]. In addition to $NAD(P)^+$-dependent enzymes catalyzing aldehyde oxidation [12,13], the ALDH family exerts properties conferring resistance (e.g., direct binding capacity [12] or UV absorption [14]) and is associated with an accelerated scavenging of reactive oxygen species [10].

The expression of cell-surface antigens associated with normal stem cells physiology is another hallmark of CSLCs [15]. CD133 is one of the most extensively employed (cancer) stem cell markers [16] and is also associated with poor prognosis of patients with non-small cell lung carcinoma (NSCLC) [17]. CD44, a receptor for hyaluronic acid, is often being combined with the cell adhesion molecule CD24 ($CD44^+/CD24^-$) for detection of CSLCs from various sources [18,19], as both antigens are involved in adhesion, migration, and metastasis [reviewed in 20].

Photodynamic therapy (PDT) represents a versatile therapeutic modality not limited to anticancer treatment. PDT utilizes a tumor-localizing photosensitizer (PS) with subsequent exposure to light. The resulting oxidizing moieties impair essential biological molecules, leading to damage or death of the target cell [21,22]. However, multiple factors affect the outcome of such a complex treatment. One of the crucial factors influencing the cytotoxic action of PDT is the intracellular level of PS, which is not linearly related to its extracellular level [23]. Therefore, it is desirable to determine all of the mechanisms that might significantly decrease the PS level prior to the actual photodynamic event. *Vice versa*, drug-light interval, that usually ranges between 24–96 h in the clinic [reviewed in 24], enables PS itself to affect the target cells and influence their susceptibility to subsequent PDT.

NSCLC accounts for the majority of all lung carcinomas [25], which is the leading and second leading cause of cancer-related mortality in men and women, respectively [26]. Today, PDT is increasingly used for the treatment of NSCLC, predominantly employed as a definitive therapy for early-stage cancers, to palliate the symptoms of advanced NSCLC or as a neoadjuvant therapy [27].

Hypericin (HYP) is considered one of the most potent naturally occurring photosensitizers yet identified [reviewed in 28]. To date, hypericin-mediated photodynamic therapy (HYP-PDT) was clinically tested exclusively for skin malignancies, and HYP holds potential as a PS for photodynamic diagnosis (PDD), especially for bladder cancer. HYP can be found in the St. John's Wort plant (SJW, *Hypericum perforatum* L.) [reviewed in 29], which is popularly used as part of custom remedies taken for self-treatment of depression. The principal reasons for SJW's popularity are ease of access and the perception of herbal remedies as safe because of their natural origin [30]. However, naturalness does not ensure inertness; on the contrary, it is well-established that the simultaneous use of extracts of SJW with other drugs, including chemotherapeutics, can decrease their effectiveness. SJW-drug interactions are known to be rendered by hyperforin, which potentiates the activity of cytochrome P450 and MDR1 [31]. However, there is also an increasing body of evidence that HYP itself, whether as a part of SJW-preparations or as a PS, could also affect the outcome of therapy via its multiple activities [reviewed in 29]. Blank has described the anticancer activities of HYP in the dark [32–34]. However, its ability to stimulate ABC transporters [35–38] can contribute to multidrug resistance (MDR).

Here, we address selected SP characteristics in the lung adenocarcinoma cell line A549 to ascertain whether SP represents a *bona fide* CSLC marker. We combined SP analysis with other CSLC markers (ALDH or CD133, CD24, and CD44 antigens) and evaluated the clonogenic, 3D-growing, and tumorigenic abilities of SP cells. Since HYP *per se* is a potential modulator of ABC transporters, we investigated HYP impact on the frequency and properties of the SP population, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

Lung adenocarcinoma A549, ovarian carcinoma A2780, promyelocytic leukemia HL-60, and the colon carcinoma cell lines SW620 and HT-29 (human origin) were obtained from the American Type Culture Collection (Rockville, MD, USA). MDR1 and MRP1 overexpressing subclones of HL-60 cells (cMDR1 and cMRP1, respectively) and the BCRP overexpressing derivative of HL-60/PLB cells (cBCRP) were kindly provided by Balazs Sarkadi, D.Sc. (Membrane Biology Research Group, Hungarian Academy of Sciences, Budapest, Hungary). cMDR1 and cMRP1 were selected by adriamycin and cBCRP were retrovirally transduced and subsequently selected by mitoxantrone [39–41].

A549 cells were cultured in Kaighn's modification of F-12 Ham Nutrient Mixture (Sigma-Aldrich, USA). All other cell lines were cultured in RPMI-1640 medium (Gibco Invitrogen Corp., USA). Both media were supplemented with 10% fetal bovine serum (FBS; Biosera, France) and antibiotics (1% Antibiotic-Antimycotic 100 × and 50 μg ml^{-1} gentamicin, Biosera). Cells were maintained at 37 °C, 95% humidity and 5% CO_2.

2.2. Experimental design

For initial experiments assessing the effect of HYP on SP percentage (%SP), A549 or A2780 cells were seeded (25×10^3 and $17 \times 10^3\,cm^{-2}$, respectively) on tissue culture dishes (TPP, Switzerland) and left to settle for 24 h. Then, HYP (HPLC grade from AppliChem GmbH, Germany) (0.1, 0.25, 0.5 or 1 μM) was added to the cells and analyses were performed 16 h later. For subsequent experiments utilizing only 1 μM HYP, A549 cells were seeded ($3 \times 10^3\,cm^{-2}$) and allowed to attach for 24 h. Subsequently, HYP was added and, 16 h later, the medium was either replaced with fresh medium (medium replacement, MR) or left for another 48 h until analyses, sorting, and processing for the clonogenic and tumorigenic test.

For experiments that did not require any treatment (SP profile, CD marker evaluation, Kinetics of intracellular accumulation of HYP and H33342 and Analysis of the intracellular HYP accumulation), A549, A2780, HT-29, and SW620 cells were seeded at different densities and allowed to attach and grow for at least 48 h to achieve 90% confluence. Leukemic cell lines HL-60, cMDR1, cMRP1, and cBCRP were seeded at the density $65 \times 10^3\,cm^{-2}$ and harvested by centrifugation 24 h later and prepared as described below.

Cells treated with HYP were always kept and processed under dark conditions to prevent undesired photoactivation.

2.3. Resazurin reduction assay

The clonogenic ability of A549 cells was estimated using the