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Cytotoxicity of natural products and derivatives toward MCF-7 cell monolayers and cancer stem-like mammospheres

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ABSTRACT

Although cancer stem-like cells (CSCs) are rare, they can enter a non-proliferative or dormant state and resist therapy. Furthermore, quiescent CSCs are responsible for metastases that can appear after curative surgical treatment of a primary tumor. Because of drug resistance of CSCs, the development of novel therapies is urgently required that specifically target CSCs.

Purpose: The aim of the present study was to investigate the potential of a panel of natural products and derivatives to inhibit CSC-enriched mammospheres of MCF-7 breast cancer cells.

Methods: $CD44^{high}/CD24^{low}$ cells were identified by flow cytometry and maintained as mammospheres. As a control, we used two clinically established anticancer drugs (5-fluorouracil and docetaxel). A panel of natural products, shikonin, two cajanin stilbene acid (CSA) derivatives and artesunate were tested by resazurin assay on CSC-enriched mammospheres and MCF-7 monolayer cells. Besides, cellular shikonin uptake experiments were performed by flow cytometry.

Results: We found two CSA derivatives (Nos. 6 and 19) to be active cancer stem-like MCF-7 mammospheres. Especially, CSA derivative No. 19 clearly showed collateral sensitivity in mammospheres compared to monolayer cells.

Conclusion: Phytochemicals which provoke collateral sensitivity in cancer-stem like cells are worth more detailed investigations in the future, since there is a great potential for improved chemotherapy to eradicate tumors and prolong cancer patients' survival times.

Introduction

Tumors are morphologically, phenotypically, and functionally heterogeneous. Therefore, multiple patterns of tumor differentiation can be distinguished (Sengupta et al. 2010). Tumors consist of cancer stem-like cells (CSCs), progenitor cells and differentiated cells, but only CSCs have the ability to generate new tumor cells due to their self-renewal and multi-lineage differentiation capability (Zhou et al. 2009; Sengupta et al. 2010). Although CSCs are rare, CSCs can initiate and maintain tumors, allow tumor propagation, colonize distant sites, and establish metastases (Sengupta et al. 2010).

Cancer generally ought not to be considered cured, even if initial responses of patients to radiation or chemotherapy lead to complete remissions. Rare CSCs are able to survive therapy, thus explaining the almost-inevitable recurrences after treatment of solid tumors by radiation or chemotherapy. Quiescent CSCs are responsible for metastases that can appear after curative surgical treatment of a primary tumor. For example, metastatic relapse in breast cancer can occur more than 10 years after initial treatment (Aguirre-Ghiso 2007; Clevers 2011). This phenomenon is called minimal residual disease. CSCs can enter a non-proliferative or dormant state and resist therapy (Patel et al. 2012). Moreover, CSCs often reside in niches composed of blood vessels, stromal cells and extracellular matrix components (Wilson and Trumpp 2006). Niches shelter CSCs from the detrimental effects of chemotherapy and favor CSC resistance (Brown et al. 1998; Green et al. 1999; Borovski et al. 2011). In addition, hypoxic areas and hypoxic tumors are radio- and drug-resistant (Brown and Wilson 2004).

One means of identifying CSCs is though cell surface markers. A subpopulation of $CD133^{high}$ cells with tumor initiating cells was observed in brain tumors (Singh et al. 2004), colon tumors (O'Brien et al. 2007; Ricci-Vitiani et al. 2007) and pancreatic cancers (Hermann et al. 2007). Furthermore, breast cancer-initiating cells with CSC-like properties were characterized by a $CD44^{high}$, $CD24^{low}$ phenotype (Al-Hajj et al. 2003). CD44 controls cell–cell interactions and

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is related to cross-talk between cancer stem-like cells and their microenvironment (Al-Hajj et al. 2003). CD24 suppresses CXCL12-CXCR4-mediated migration of cells. Therefore, loss of CD24 expression favors invasion and metastasis (Schabath et al. 2006). These breast cancer-initiating cells were considered to have stem cell-like properties, because they reconstituted the heterogeneity of the originating tumors. Breast cancers with HER2/neu amplification and CSC features ($CD44^{high}$, $CD24^{low}$) are resistant to neoadjuvant chemotherapy and HER2 pharmacological inhibition (Li et al. 2008). Because of CSC drug resistance, the design of novel therapies to specifically target CSCs is urgently required.

Natural products play a crucial role in cancer research, since many are currently used in the clinic. More than two thirds of anticancer drugs approved between the 1940s and 2006 are natural products or were developed on the basis of natural products. Well known examples include *Vinca* alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), epipodophyllotoxines (etoposide, teniposide), camptothecine and its derivatives (topothecan, irinothecan), and anthracyclines (doxorubicin, daunorubicin, epirubicine, idarubicin).

Therefore, it comes as no surprise that cytotoxic compounds from plants and microorganisms are a valuable resource for drug development (Efferth 2010). Natural products have also been described to inhibit CSCs (Efferth 2012). Given the fact that CSCs are resistant toward standard chemo- and radiotherapy, natural compounds may bear potential to eradicate CSCs with similar efficacy as they eradicate the general tumor population.

The purpose of the present investigation was to investigate the potential of a panel of natural products and derivatives to inhibit CSC-enriched mammospheres of MCF-7 breast cancer cells. As a control, we used two clinically established anticancer drugs (5-fluorouracil and docetaxel). $CD44^{high}/CD24^{low}$ cells, which are considered CSCs (Guttilla et al. 2012), were identified by flow cytometry and maintained as mammospheres to better mimic the three-dimensional growth of tumors. These CSC-enriched mammospheres were compared with MCF-7 monolayer cells grown under standard conditions.

Material and methods

Chemicals

5-Fluorouracil (PubChem CID:3385) (5-FU) was provided by the University Medical Center of the Johannes Gutenberg University (Mainz, Germany) and dissolved in PBS (Invitrogen, Eggenstein, Germany) at a concentration of 50 mg/ml.

Shikonin (PubChem CID:479503) (purity ≥98%, HPLC grade; Fig. 1) (**3**) was obtained from Enzo Life Sciences (Lausen, Switzerland) and 50 mM stock solutions were prepared in DMSO.

Two cajanin stilbene acid (CSA) derivatives (No. 6 and No. 19) (**2**, **1**) (purity ≥98%, HPLC grade) were kindly provided by one of the authors (YJF) (Fig. 1). All CSA derivatives were dissolved in DMSO (Sigma–Aldrich, Germany) for preparation of 100 mM stock solutions.

Artesunate (PubChem CID:5464098) (purity ≥99%, HPLC grade, Fig. 1) (**4**) was purchased from Saokim Ltd. (Hanoi, Vietnam) and 60 mM stocks were prepared in DMSO.

Cell culture

Monolayer cells. The MCF-7 cell line was obtained from the German Cancer Research Center (DKFZ, Heidelberg, Germany). The original source of the cell line is the American Type Culture Collection (ATCC, USA). MCF-7 cells were incubated in DMEM medium with GlutaMAX (Invitrogen, Germany) supplemented with 10% FBS and 1% penicillin (100 U/ml)–streptomycin (100 μg/ml). Cells were maintained in a humidified environment at 37 °C with 5% CO_2 and sub-cultured twice per week. All experiments were performed on cells in the logarithmic growth phase.

Mammosphere formation

To form mammospheres, MCF-7 cells were plated in 25 cm^2 culture flasks coated with 20 mg/ml polyHEMA at density of 2×10^5 cells/ml in DMEM/F12 medium supplemented with fibroblast growth factor (10 ng/ml), epidermal growth factor (10 ng/ml), B27 supplement (100 units/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). After culturing for 1 week, spheres were visible by inverted phase-contrast microscopy, and these spheres were defined as mammospheres in passage 1 (MS P1). Prolonged mammosphere culture was achieved using weekly trypsinization and dissociation with a cell strainer followed by reseeding in mammosphere media into polyHEMA treated culture flasks. Each generation was designated by the week of culture (MS P1, MS P2, etc.). After 5 weeks mammospheres were dissociated by trypsinization and returned to standard adherent culture conditions in serum-supplemented DMEM media. Cells were culture for 1 week and subsequently used for further experimentation.

CD24/CD44 phenotype enrichment assay

MCF-7 mammospheres were treated with 0.25% trypsin/EDTA solution to obtain single cell suspensions. Subsequently, cells were washed twice with staining buffer (DPBS + 5% FBS) and the cell number was adjusted to 1×10^7 cells/ml. Twenty microliters of PE mouse anti-human CD44 antibody and FITC mouse anti-human CD24 antibody (both BD Pharmingen) were added to 100 μl of cell suspension (1×10^6 cells) and incubated in the dark on ice for 20 min. After antibody incubation, cells were washed twice with staining buffer and finally resuspended in 500 μl staining buffer. Subsequently, cells were measured in an LSR-Fortessa FACS analyzer (Becton-Dickinson). For each sample, 1×10^4 cells were counted. The PE signal was measured at 561 nm excitation and detected using a 586/15 nm band pass filter. The FITC signal was analyzed at 488 nm excitation and detected using a 530/30 nm band pass filter. All parameters were plotted on a logarithmic scale. Cytographs were analyzed using FlowJo software (Celeza).

Resazurin reduction assay

A resazurin reduction assay was used to investigate cytotoxicity of several drugs toward MCF-7 monolayer and mammosphere cells. The assay is based on reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Nonviable cells rapidly

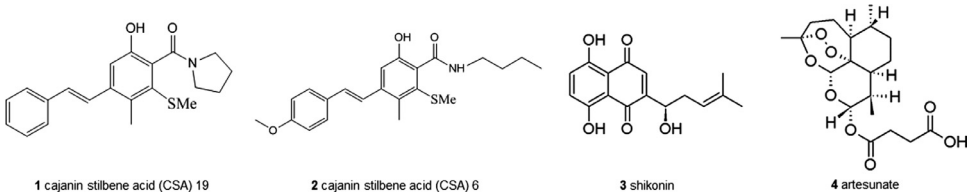

Fig. 1. Chemical structures of investigated compounds.