Biochemical Pharmacology 93 (2015) 210–220

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Alternation of adriamycin penetration kinetics in MCF-7 cells from 2D to 3D culture based on P-gp expression through the Chk2/p53/NF-κB pathway

Meng Lu [a,1], Fang Zhou [a,b,1], Kun Hao [a,b], Jiali Liu [a], Qianying Chen [a], Ping Ni [a], Honghao Zhou [c], Guangji Wang [a,b,*], Jingwei Zhang [a,b,*]

[a] Key Laboratory of Drug Metabolism and Pharmacokinetics, State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, Jiangsu, China
[b] Jiangsu Key Laboratory of Drug Design and Optimization, China Pharmaceutical University, Nanjing, Jiangsu, China
[c] Institute of Clinical Pharmacology, Central South University, Changsha, Hunan, China

ARTICLE INFO

Article history:
Received 20 September 2014
Accepted 19 November 2014
Available online 3 December 2014

Chemical compounds studied in this article:
Adriamycin (PubChem CID: 31703)
LY335979 (PubChem CID: 153997)
AZD7762 (PubChem CID: 11152667)
Nutlin-3 (PubChem CID: 216345)
DMSO (PubChem CID: 679)
Propidium iodide (PubChem CID: 104981)
MTT formazan (PubChem CID: 16218671)

Keywords:
Multi-cellular layer
Penetration kinetics
P-gp
Chk2/p53/NF-κB pathway

ABSTRACT

Monolayer cells are largely different from tumor masses, and might misguide drug screenings. 3D *in vitro* cell culture models simulate the characteristics of tumor masses *in vivo* and have recently been used in many studies of anti-cancer drugs. Among various 3D cell culture models, multi-cellular layer (MCL) models allow for the direct quantitative assessment of the penetration of chemotherapeutic agents through solid tissue environments without requiring the use of fluorescently labeled drugs or imaging molecules. Therefore, in our present study, a 3D-no base and embedded MCF-7 MCL model was successfully developed for a 14-day culture. Over time, its thickness and cell layers increased and exhibited highly proliferative properties and drug resistance to adriamycin (ADR) with markedly elevated IC_{50} values. Meanwhile, G2/M stage cycle arrest was also observed, which likely up-regulated P-gp expression through the Chk2/p53/NF-κB pathway. The elevated P-gp expression altered the ADR penetration kinetics in MCF-7 MCLs *in vitro* by accelerating the apparent penetration of ADR through the intercellular spaces of the MCLs. Additionally, a decreased ADR retention within tumor cells was observed, but could be significantly reversed by a P-gp inhibitor. The attenuated ADR retention in the deeper cells of tumor masses was confirmed in xenografted mice *in vivo*. This phenomenon could be elucidated by the mathematical modeling of penetration kinetics parameters. Our study provided a new model that evaluated and improved the quantification of the drug penetration kinetics, revealed the relationship between P-gp and drug penetration through tumor masses, and suggested the potential molecular mechanisms.

1. Introduction

In order to achieve therapeutic efficacy, sufficient concentrations of anti-cancer drugs should thoroughly penetrate tumor masses to gain full access to all viable cancer cells. However, most conventional anti-cancer drugs are confined to only the periphery of tumor masses near the vasculature [1,2], a phenomenon that is known as multi-cellular resistance (MCR) [3,4]. This is one of the main reasons for treatment failure of anti-cancer drugs. The many mechanisms responsible for MCR are interrelated and multi-faceted [5,6] and include the over-expression of efflux pumps through cytokine alternations [7] and cell cycle changes [8]. Therefore, new anti-cancer agents that account for the spatial structure of tumor masses should be developed. However, *in vitro* cultures of cell monolayers are still the most common choice in conventional studies, and these differ from physiological tumor masses. The majority of anti-cancer agents has been tested on cell monolayers and may thus provide results and interpretations that are not applicable for clinical use.

Abbreviations: ADR, adriamycin; AUC, area under the concentration–time curve; ECM, extracellular matrix; ESI, electrospray ionization; HBSS, Hank's balanced salt solution; LC–MS/MS, liquid chromatography tandem mass spectrometry; MCL, multi-cellular layer; MCR, multi-cellular resistance; MCS, multi-cellular spheroid; MDR, multi-drug resistance; MRM, multiple reaction monitoring; P-gp, P-glycoprotein; PI, propidium iodide; qPCR, quantitative real-time PCR; S.E., standard error.

* Corresponding authors.
E-mail addresses: guangjiwang@hotmail.com (G. Wang), zhangjw_cnnj@sina.com (J. Zhang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bcp.2014.11.010
0006-2952/

Due to the non-physiological environments represented by monolayer cell cultures, various 3D cell culture models have been developed for more accurate drug evaluations. Multi-cellular layer (MCL) and multi-cellular spheroid (MCS) systems are two important models that can reproduce the characteristics of tumor masses *in vivo* and have been used widely in many pharmacokinetic and pharmacodynamic studies of anti-cancer drugs [9,10]. MCS systems are believed to more accurately represent the structures and biochemical properties of tumor masses than do MCL systems [11,12]. However, for kinetics analyses, MCS systems have their limitations. Accurate drug concentrations cannot be determined at different depths within a cell spheroid or can only be achieved using semi-quantitative fluorescence-based imaging [13–15]. MCL models provide for the direct quantitative assessment of the penetration of chemotherapeutic agents through solid tissue environments without the need for fluorescent labeling. Thus, MCL models can be used to obtain the necessary information on drug penetration and distribution properties through multi-cell barriers for optimizing drug delivery [16–18].

In our previous studies, we analyzed the cellular pharmacokinetic mechanisms of multi-drug resistance (MDR) of breast cancer cell MCF-7 monolayers induced by the anti-cancer agent, adriamycin (ADR). Additionally, we screened for an effective MDR reversal agent, ginsenoside Rh2 [19]. However, because breast cancer is a solid tumor and presents MCR, there is a pressing need to analyze the penetration kinetics of anti-cancer agents and explore strategies to optimize tumor tissue penetration. Therefore, MCF-7 MCL models were developed in our present study. The penetration kinetics of ADR on MCF-7 monolayers and MCL models were investigated, compared and mathematically modeled. Xenograft *in vivo* tumor models were also performed for confirmation. Finally, the MCR of MCF-7 MCL models and its pharmacokinetic-related mechanisms were tentatively elucidated.

2. Materials and methods

2.1. Materials

Adriamycin (purity > 99%) was purchased from Shenglin Chemical industry (Jiangsu, China). LY335979 and AZD7762 were purchased from Selleckchem (Houston, Texas, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide (PI), Nutlin-3, DMSO, HPLC-grade methanol and formic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Deionized water was prepared by a Milli-Q system (Merck Millipore, Billerica, MA, USA) and was used throughout. Matrigel, FITC-conjugated anti-P-gp antibody and isotype antibody were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Standard cell culture inserts (CM 6.5 mm, pore size 0.4 μm) were purchased from Merck Millipore (Billerica, MA, USA). Monoclonal antibodies against Chk2, p-Chk2, p53, p65, Lamin B, and horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody for GAPDH was purchased from Bioworld Technology (Dublin, Ohio, USA). The SYBR Prime Script RT-PCR Kit was purchased from Takara Bio Inc. (Otsu, Shiga, Japan).

2.2. Animal welfare and ethical statements

Healthy female BALB/c nude mice (18–22 g and 8–10 weeks old) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and were kept ten per cage at room temperature (22 ± 1 °C) with 50–60% relative humidity and automatic day–night rhythm (12 h-cycle) in an SPF-grade environment. Tumors were generated by subcutaneous injections of 5×10^6 exponentially growing MCF-7 cells into the right flank regions of female athymic nude mice. Estrogen pellets were implanted into the nude mice one day before injection of cells. Prior to each experiment, the animals were fasted overnight (12 h) with free access to water. All animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University (Nanjing, Jiangsu, China). This study was carried out in strict accordance with the Guidelines for Animal Experimentation of this institution. All procedures were as humane as possible. Every effort was made to minimize animal pain, suffering and distress and to reduce the number of animals used.

2.3. Monolayer cell culture

MCF-7 human breast carcinoma cells were purchased from American Type Culture Collection. Cells were grown in monolayers using RPMI 1640 medium supplemented with 10% fetal bovine serum, and 100 U ml^{-1} penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA). The cells were grown in an atmosphere of 5% CO_2 at 37 °C, and cell medium was changed every other day.

2.4. Multi-cellular layer culture

The multi-cellular layer (MCL) culture of MCF-7 cells was developed according to a "3D-no base and embedded" model with minor modifications [20]. Briefly, cells were suspended in serum-free medium containing 6% matrigel and seeded onto the uncoated cell culture inserts. After incubating at 37 °C for 1 h to ensure matrigel solidification, cell culture media was added. The cells were further incubated at 37 °C to form MCLs; subsequently, the medium was changed daily.

2.5. Histology assays and packing density measurements

Cells were fixed in 10% neutral-buffered formalin for 24 h, processed with gradient concentrations of ethanol, placed in xylene overnight and subsequently, embedded in paraffin. Sections were cut at a thickness of 4 μm and stained with H&E and Ki-67. The thickness of the MCL was determined with a microscope (Leica, Wetzlar, Germany). The packing density was calculated as the percentage of nuclear areas in all areas of MCF-7 cells. The number of nuclei per unit surface area in each image was quantified using Leica Qwin Lite.

2.6. Cell cycle analysis

The cell cycle distribution was assayed by determining the DNA content of MCF-7 cells. Cells were fixed in 70% ethanol overnight at 4 °C, and re-suspended in a staining solution containing RNase A and propidium iodide (PI) for 30 min. After washing, the DNA content was determined by flow cytometry (FACS Calibur, BD, Franklin Lakes, New Jersey, USA) and analyzed with CELLQUEST software.

2.7. Cytotoxicity assay

MCF-7 cells were exposed to various concentrations of ADR for 72 h at 37 °C with 5% CO_2. The cell sensitivities were determined by measuring cell growth inhibition *via* MTT colorimetric assay. The IC_{50} values were calculated from survival curves using the Bliss method.

2.8. Quantitative real-time PCR assay

The quantitative real-time PCR (qPCR) reactions were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Premix Ex Taq II (Takara, Otsu, Shiga, Japan). The primers were synthesized by Invitrogen