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Interrogation of Multidrug Resistance (*MDR1*) P-glycoprotein (Pgp, ABCB1) Expression in Human Pancreatic Carcinoma Cells: Correlation of ^{99m}Tc-Sestamibi Uptake with Western Blot Analysis

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Abstract

Objective—Histopathological studies indicate approximately 63% of pancreatic tumors express *MDR1* Pgp and its polymorphic variants. However, Pgp expression detected at the messenger RNA or protein level does not always correlate with functional transport activity. Because Pgp transport activity is affected by specific mutations as well as the phosphorylation state of the protein, altered or less active forms of Pgp may also be detected by PCR or immunohistochemistry, which do not accurately reflect the status of tumor cell resistance. To interrogate status of functional expression of *MDR1* Pgp in MiaPaCa-2 and PANC-1 cells, cellular transport studies using ^{99m}Tc-Sestamibi were performed and correlated with western blot analysis.

Methods—Biochemical transport assays in human pancreatic carcinoma MiaPaCa-2 and PANC-1 cells, human epidermal carcinoma drug sensitive KB-3-1 cells and human breast carcinoma MCF-7 cells (negative controls), and human epidermal carcinoma drug resistant KB-8-5 cells, human breast carcinoma stably transfected with Pgp MCF-7/*MDR1*Pgp cells, and liver carcinoma HepG2 cells (positive controls) were performed. Protein levels were determined using a monoclonal antibody C219.

Results—^{99m}Tc-Sestamibi demonstrates accumulation in human pancreatic carcinoma MiaPaCa-2 and PANC-1 cells. Uptake profiles are not affected by treatment with LY335979, a Pgp-inhibitor, and correlate to Western blot analysis.

Conclusions—These cellular transport studies indicate an absence of Pgp at a functional level in MiaPaCa-2 and PANC-1 cells. Because major pancreatic tumors originate from pancreatic duct and ^{99m}Tc-Sestamibi undergoes a dominant hepatobiliary mode of excretion, it would not be a sensitive probe for imaging pancreatic adenocarcinomas. Following interrogation of the functional

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status of Pgp in other pancreatic carcinoma cells, chemotherapeutic drugs that are also *MDR1* substrates could offer alternative therapeutics for treating pancreatic adenocarcinomas.

INTRODUCTION

Pancreatic cancer is among the deadliest of cancers and is the fourth most common cause of sudden death in the United States [1]. Due to a lack of specific symptoms and limitations in diagnostic techniques, the disease often eludes detection during its initial stages. To enable diagnosis, various clinical biomarkers, such as serum markers, sialylated Lewis blood group antigen (CA19-9) [2, 3], DNA methylation alterations [4], and genetic signatures (K-ras, p53 mutations) [5] have been identified. Among the well-characterized drug resistance pathways in cancer chemotherapy, histopathological studies have shown that 63% of pancreatic tumors express *MDR1* Pgp [6] and its polymorphic variants [7]. Additionally, recent reports also indicate the presence of multidrug resistance (*MDR1*) P-glycoprotein (Pgp; ABCB1) in human pancreatic carcinoma cells, such as MiaPaCa-2 and PANC-1, following treatment with low doses of vincristine [8], thus suggesting the ability of therapeutic drugs to induce drug resistance. These factors mandate development of chemotherapeutics capable of overcoming transporter-mediated efflux pathways and also noninvasive imaging methodologies for interrogating the functional status of Pgp in pancreatic tumors.

To address these objectives, interrogation of a Pgp-mediated functional transport could serve as an important marker for stratification of patients likely to benefit from a given chemotherapeutic treatment. Furthermore, a new generation of taxoids have been developed to overcome drug-resistance pathways mediated by Pgp and are further postulated to be the second generation chemotherapeutics with minimal side-effects compared to taxanes [9]. Additionally, these molecules show potent activity against human pancreatic carcinoma MiaPaCa-2 and PANC-1 cells, that show expression of Pgp (ABCB1) and other drug-resistance proteins, such as MRP1 (ABCC1) and MRP2 (ABCC2), based upon reverse transcription polymerase chain reaction (RT-PCR) [9]. However, Pgp expression, as detected at the messenger RNA [10] or protein level, does not always correlate with functional Pgp-mediated transport activity. Because Pgp transport activity is affected by specific mutations as well as the phosphorylation state of the protein [11–13], altered or less active forms of Pgp may also be detected by PCR or immunohistochemistry, which do not accurately reflect the status of tumor cell resistance.

Thus, methods to interrogate Pgp transport activity have been long sought [14]. Imaging with a radiopharmaceutical that is transported by Pgp may identify noninvasively the tumors in which the transporter is not only expressed, but is also functional [15]. Although numerous radiopharmaceuticals have been characterized for probing Pgp-mediated transport *in vitro* and *in vivo*, imaging agents, such as ^{99m}Tc-Sestamibi, ^{99m}Tc-Tetrofosmin, and ^{67/68}Ga-ENBDMPI represent, highly sensitive agents for monitoring its transport function [14] *in vivo*. These agents share common features, such as moderate hydrophobicity and a delocalized positive charge on the surface, allowing penetration into cells (Pgp-) in response to favorable negative plasma- and mitochondrial transmembrane potentials [16]. Herein we report the ability of ^{99m}Tc-Sestamibi, a highly sensitive radiopharmaceutical, to

assess functional expression of Pgp on the plasma membrane of human MiaPaCa-2 and PANC-1 pancreatic carcinoma cells and correlate results via Western blot analysis. Overall, this methodology could be employed to interrogate functional status of Pgp in pancreatic carcinoma cells.

EXPERIMENTAL

General Methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Radiochemistry

Preparation of ^{99m}Tc -Sestamibi— ^{99m}Tc -Sestamibi (Triad Isotopes) was loaded onto a C-18 Sep-Pak cartridge (Waters), which had been primed first with 90/10 ethanol/saline (3mL), followed with saline (5mL), and air (5mL) to remove residual liquid. Further, the cartridge containing ^{99m}Tc -Sestamibi was washed with saline (10mL), and the residual saline was also excluded with air (10mL). Finally, ^{99m}Tc -Sestamibi was eluted from the cartridge with 90/10 ethanol/saline (2mL) [17, 18].

Biochemistry

Cell Culture—Monolayers of cells were grown at 37°C under a humidified 5% CO_2 atmosphere. Human epidermoid carcinoma non-MDR KB-3-1 (Pgp-) and both pancreatic carcinoma MiaPaCa-2 and PANC-1 cells were grown in high glucose DMEM (GIBCO, 11965) supplemented with heat-inactivated fetal bovine serum (10%) and L-glutamine (2mM). Human epidermoid carcinoma colchicine-derived MDR KB-8-5 cells (Pgp+) were grown in high glucose DMEM supplemented with heat-inactivated fetal bovine serum (10%), L-glutamine (2mM), and colchicine (10ng/mL). Pgp-transfected human breast cancer MCF-7/*MDR1* Pgp cells (Pgp+) and their control MCF-7 cells (Pgp-) were grown in high glucose DMEM supplemented with heat-inactivated fetal bovine serum (10%), L-glutamine (2mM), and G418 (1mg/mL). Human liver carcinoma HepG2 cells were grown in high glucose DMEM supplemented with heat-inactivated fetal bovine serum (10%).

Cellular Accumulation—Cellular transport of ^{99m}Tc -Sestamibi was determined in 24-well tissue culture treated plates. Cells (approximately 50,000/well) were plated in media (high glucose DMEM supplemented with heat-inactivated fetal bovine serum (10%) and L-glutamine (2mM)) and allowed to recover overnight. Media were removed from cells and replaced with media containing the desired concentrations of ^{99m}Tc -Sestamibi (74kBq/mL), in the presence or absence of Pgp inhibitor LY335979 (1 μM). Cells were incubated under normal growth conditions (37°C, 5% CO_2 atmosphere) for 90min, and then washed 3 $\times$ with 4°C DPBS (Dulbecco's phosphate buffered saline lacking CaCl_2 and MgCl_2). Cells were then extracted in 1% SDS (sodium dodecyl sulfate) with 1 mM sodium borate. Aliquots of the loading solution and ^{99m}Tc -Sestamibi stock solutions also were obtained for standardizing cellular data with the extracellular concentration of ^{99m}Tc -Sestamibi. All cell extracts, ^{99m}Tc -Sestamibi stock solutions, and loading solution samples were assayed for γ -activity in a well-type sodium iodide γ -counter (Cobra II; Packard). Protein concentration of cell extracts was determined by the BCA (bicinchoninic acid) assay (Pierce Chemical Co.,

Rockford, IL), using bovine serum albumin as the protein standard. Data are reported as $\text{fmol } ^{99\text{m}}\text{Tc-Sestamibi (nM}_0\text{)}^{-1} (\text{mg protein})^{-1}$, with nM_0 representing the total concentration of $^{99\text{m}}\text{Tc-Sestamibi}$ in the extracellular buffer.

Western Blot Analysis

Cells were cultured in 35mm dishes, then harvested and lysed in RIPA buffer (150mM NaCl, 50mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4), supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), sodium orthovanadate (1mM), and PMSF (1mM). Whole-cell lysates were normalized for protein content by BCA assay (Pierce Chemical Co., Rockford, IL). Proteins were resolved by SDS-PAGE (4%-15%, Biorad Criterion Tris-HCl, Hercules, CA), transferred to a PVDF membrane (Hybond-P, Amersham, UK), and probed with anti-Pgp antibody (C219, Abcam, Cambridge, UK) and anti-actin antibody (data not shown, A2066, Sigma, St. Louis, MO). Following treatment with secondary horseradish peroxidase-IgG antibodies (GE Healthcare Biosciences, Piscataway, NJ), the blots were imaged in chemiluminescent solution (GE Healthcare, Amersham ECL Western Blotting Detection Reagents, RPN2209) on an IVIS 100 (Xenogen, Caliper Life Sciences). Densitometry analysis was performed with Living Image 3.2 (Xenogen, Caliper Life Sciences) and Excel (Microsoft) software.

RESULTS AND DISCUSSION

Previously, our lab, as well as others, has shown that moderately hydrophobic cationic compounds possessing a delocalized positive charge on their surface penetrate Pgp-lacking human carcinoma (non-MDR) cells in response to favorable negative plasma- and mitochondrial-transmembrane electrochemical gradients and localize into mitochondria [16, 19, 20]. Among the numerous molecules recognized by Pgp as its transport substrates, $^{99\text{m}}\text{Tc-Sestamibi}$ is a clinically useful, ultrasensitive, and thoroughly investigated molecular imaging probe for monitoring functional status of the Pgp *in cellulo* and *in vivo*. While performing biochemical transport assays in human pancreatic carcinoma MiaPaCa-2 and PANC-1 cells, we employed human epidermal carcinoma drug sensitive KB-3-1 cells and human breast carcinoma MCF-7 cells as negative controls, and human epidermal carcinoma drug resistant KB-8-5 cells, human breast carcinoma stably transfected with Pgp MCF-7/*MDR1*Pgp cells, and liver carcinoma HepG2 cells as positive controls. The stably transfected MCF-7/*MDR1* Pgp cells provide a model transport system without the possible confounding effects of other drug-resistance pathways, which could be induced during selection of cells in the presence of cytotoxic agents. Overall, cellular accumulation data demonstrate that uptake profiles of $^{99\text{m}}\text{Tc-Sestamibi}$ are inversely proportional to Pgp expression, and LY335979, a highly specific and potent inhibitor of Pgp, induces uptake of the lipophilic cation in the Pgp expressing MDR cells (Figure 1). Importantly, $^{99\text{m}}\text{Tc-Sestamibi}$ shows accumulation in MiaPaCa-2 and PANC-1 cells. Additionally, incubation of these cells with LY335979 does not significantly impact uptake profiles of $^{99\text{m}}\text{Tc-Sestamibi}$, indicating the absence of functional Pgp. To further correlate these data, we performed Western blot analysis of cell lysates using a Pgp specific monoclonal antibody, C219. Compared with positive controls (KB-8-5, MCF-7/*MDR1*Pgp, HepG2) showing abundant immuno-detectable Pgp, human pancreatic carcinoma MiaPaCa-2 and PANC-1 cells did not

show significant expression of Pgp (Figure 2), thus consistent with cellular uptake profiles of ^{99m}Tc -Sestamibi in MiaPaCa-2 and PANC-1 cells.

Overall, these data indicate that ^{99m}Tc -Sestamibi could be employed as a molecular imaging probe to assess functional status of Pgp in pancreatic carcinoma cells. Finally, following interrogation of functional status of Pgp in other pancreatic carcinoma cells, chemotherapeutic drugs that are also *MDR1* substrates could also offer alternative therapeutics for treating pancreatic adenocarcinomas.

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Figure 1. Cellular Accumulation Profiles

Cellular accumulation of ^{99m}Tc -Sestamibi in human epidermal carcinoma (KB3-1), breast carcinoma MCF-7, Pgp⁻ controls; human epidermal drug resistant (KB-8-5), *MDR1* Pgp transfected breast carcinoma (MCF-7/*MDR1*), liver carcinoma HepG2, Pgp⁺ controls; and pancreatic carcinoma (PANC-1, MiaPaCa-2) cells. Shown is net uptake at 90min ($\text{fmol} * \text{nM}_0^{-1} * \text{mg Protein}^{-1}$) using control buffer in the absence and presence of an *MDR1* Pgp inhibitor (LY335979).

Figure 2. Western Blot analysis of KB-3-1, MCF-7, Pgp⁻ controls; KB-8-5, MCF-7/MDR1, HepG2, Pgp⁺ controls; and PANC-1, MiaPaCa-2 cells using an anti-Pgp monoclonal antibody (C219). The 170kDa band corresponds to Pgp.