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Tumor microenvironment determines response to a heat-activated thermosensitive liposome formulation of cisplatin in cervical carcinoma

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ABSTRACT

Significant heterogeneity in the tumor microenvironment of human cervical cancer patients is known to challenge treatment outcomes in this population. The current standard of care for cervical cancer patients is radiation therapy and concurrent cisplatin (CDDP) chemotherapy. Yet this treatment strategy fails to control loco-regional disease in 10–30% of patients. In order to improve the loco-regional control rate, a thermosensitive liposome formulation of CDDP (HTLC) was developed to increase local concentrations of drug in response to mild hyperthermia (HT). The HTLC formulation in combination with local HT demonstrated a significant therapeutic advantage in comparison to free drug and Lipoplatin™ in ME-180 and SiHa xenograft models of human cervical cancer, as well as in four distinct cervical patient-derived xenograft models. Differential response to HTLC + HT treatment was observed between the ME-180 and SiHa tumor models. Tumor doubling time, *in vitro* cell sensitivity, and tumor drug accumulation were found to be non-predictive of treatment efficacy. Rather, tumor microenvironment parameters, in particular elevated levels of both tumor hypoxia and associated stromal content, were found to serve as the overriding factors that limit drug efficacy. The prognostic value of these markers may enable stratification of cervical cancer patients for implementation of personalized medicine in the clinical setting.

1. Introduction

Tumor microenvironment (TME) is composed of both malignant cells and tumor-associated stroma. The major components of tumor-associated stroma, blood vessels, immune cells, cancer-associated fibroblasts (CAFs), and extracellular matrix (ECM), are responsible for its structural integrity, transport of essential molecules, inflammatory response and waste removal [1]. Remodeling and production of ECM by CAFs is often deregulated in tumor tissue [2]. Tumor-associated stroma, in particular the ECM, poses a physical barrier to intratumoral drug penetration limiting the efficacy of drug treatment. Indeed, compelling evidence supports the role of tumor-associated stroma in disease progression and poor clinical outcomes [3].

Hypoxia, a known indicator of tumor aggression, is another significant obstacle to drug therapy [4–6]. Tumors can engage in vascular formation, beyond their metabolic requirement, as a means to support uncontrolled proliferation and invasion [7]. The aberrations in tumor vascular development lead to limited blood perfusion and hypoxic regions within the tumor that are characterized by low oxygen partial pressure (pO_2) [8]. In the case of cervical cancer, considerable heterogeneity in tumor-associated stromal content [9,10] and oxygen partial pressure (pO_2) [11,12] has been observed in patient tumor tissues. In human cervical tumors, pO_2 values have been shown to range from 0 to 100 mmHg with a mean value of 15 mmHg [11,12], with tumor-associated stromal content ranging from 6% to 82% with a mean value of 43% [9].

In the past decade, significant improvements have been made in the outcomes for patients diagnosed with cervical cancer, in particular for

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those with early stage disease [13]. The current standard of care for patients with cervical cancer is radiation therapy and concurrent cisplatin (CDDP) chemotherapy. However, this treatment approach fails to control the loco-regional disease in 10–30% of patients [14]. In a recent clinical trial, conducted in patients with cervical cancer at the Princess Margaret Cancer Center (PMH, Toronto, Canada), the addition of concurrent CDDP to radiation therapy resulted in a significant improvement in overall survival (70% *vs.* 57%, HR 0.66, $p = 0.036$) [15].

With the aim of developing a strategy to further improve the loco-regional control rate in cervical cancer patients, a thermosensitive liposome formulation of CDDP (HTLC) was developed to provide heat-activated, site-specific release of drug at the primary tumor site [16,17]. The development of thermosensitive liposomes with an ultrafast drug release profile (*i.e.* ThermoDox®) was led by Needham and Dewhirst as a means to improve tumor drug availability of conventional non-thermosensitive liposome formulations and to reduce the systemic toxicity associated with free drug [18,19]. ThermoDox® is currently in clinical development and other promising thermosensitive liposome formulations of drug are in late stage preclinical development [16,20–24]. We previously reported that HTLC in combination with local hyperthermia (HT) resulted in a significant increase in tumor accumulation of drug and tumor growth delay in an ME-180 xenograft model of human cervical cancer in comparison to free drug when administered at chemically equivalent doses of CDDP [16]. Given the short-circulation lifetime of the HTLC formulation, a heating protocol that included 5 min heating prior to administration of HTLC and 20 min heating post-injection was employed to maximize intravascular release of drug from HTLC upon first pass through the heated tumor region. The intravascular release of drug from the formulation creates a substantial concentration gradient that serves as a driving force to promote drug entry into the tumor interstitium. As well due to the high aqueous solubility and limited cell membrane permeability of CDDP (log $P = -2.19$ to -2.53) [25–27], it is postulated that a fraction of the drug remains in the systemic circulation. Systemic drug can be advantageous for treating metastatic lesions in addition to tumor at the primary site.

Given the highly variable and challenging TME present in human cervical cancer, the current study aims to elucidate the impact of TME parameters on treatment response to the HTLC formulation, free drug and a non-thermosensitive liposome formulation of CDDP (*i.e.* Lipoplatin™) with or without (+/−) HT when administered at their maximum tolerated doses (MTDs). In order to investigate this, the study was conducted in two distinct xenograft models established from two immortalized cell lines (*i.e.* ME-180 and SiHa) of human cervical cancer that vary significantly in TME parameters, in particular tumor-associated stroma and hypoxia.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DPPG, Na), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (MSPC or S-lyso-PC) and *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt ($mPEG_{2000}$-DSPE) were purchased from Corden Pharma Switzerland LLC (Liestal, Switzerland). Platinum standard solution and *cis*-diamineplatinum (II) dichloride (CDDP) were obtained from Sigma-Aldrich (Oakville, ON). 0.9% saline, fetal bovine serum (FBS) and 10% formalin solution were bought from MedStore at the University of Toronto (Toronto, ON). Lipoplatin™ was generously provided by Regulon Inc. (Athens, Greece). Alpha-minimal essential medium (α-MEM) was purchased from Tissue Culture Media Facility at University Health Network (UHN, Toronto, ON).

2.2. Formulation preparation and characterization

Details regarding the preparation procedures of the HTLC formulation were described in our previous publications [16,28]. The hydrodynamic diameter of the HTLC formulation was measured using a Malvern Zetasizer instrument (Malvern, UK) based on dynamic light scattering (DLS). The CDDP solution was prepared by dissolving CDDP in 0.9% of saline at a concentration of 1 mg/mL. The platinum concentration in the HTLC liposomes, CDDP solution and Lipoplatin™ liposomes was quantified using inductively coupled plasma-atomic emission spectrometry (ICP-AES Optima 7300DV, PerkinElmer, Waltham, MA). Preparation of the HTLC formulation was found to be highly reproducible with consistent results obtained from batch to batch for the physiochemical properties of the formulation. In brief, the phase transition temperature, drug concentration, size and zeta potential of the HTLC formulation were found to be 41.5 ± 0.5 °C, 1.0 ± 0.2 mg/mL, 110 ± 9 nm and − 31 ± 2 mV, respectively [16].

2.3. Animals, tumor models and HT protocol

Female mice with severe combined immunodeficiency (SCID) were purchased from an in-house breeding facility at the UHN (Toronto, ON). All animal studies were conducted under protocols approved by the Animal Resources Center (ARC, AUP #3481) and Research Ethics Board (REB, 11-0299-CE) at UHN.

The human cervical carcinoma cell lines, ME-180 and SiHa, were kindly provided by Dr. Richard P. Hill's laboratory (UHN, Toronto, ON). Cell line authentication was not routinely performed. Both cell lines were cultured in α-MEM supplemented with antibiotics and 10% FBS and maintained in a 5% CO_2 incubator. Procedures regarding tumor inoculation and implantation of subcutaneous ME-180 and SiHa tumor models were previously described [16]. Specifically, 1×10^6 ME-180 or SiHa cells were inoculated intramuscularly into the hind limb of female SCID mice (*i.e.* donor mice). Tumors grown in donor mice were excised and cut into cubic fragments of approximately 2–3 mm^3. Each tumor fragment was then implanted subcutaneously into the left hind limb of a recipient female SCID mouse. Tumor growth was assessed according to caliper measurement of changes in tumor width (w) and length (l). Tumor volume was calculated based on the formula $V = \pi/6(w^2)(l)$. ME-180 and SiHa tumors were allowed to grow to approximately 90 mm^3 prior to use of animals in any *in vivo* studies.

The four patient-derived xenograft (PDX) models employed in this research were previously developed by the laboratories of Drs. Hill and Milosevic at the PMH [29,30]. Studies involving human samples were approved by the REB at UHN. In brief, the PDX models were derived from biopsies removed from different cervical cancer patients prior to treatment. Patient cervix tissue biopsies were collected from a PMH clinic and transported in a sealed container. Biopsies with sample numbers 28, 29, 30 and 34 were evaluated in the present study [31]. Each biopsy tissue was cut into small fragments of approximately 2–3 mm^3 and one piece of the fragment was implanted subcutaneously into a female SCID mouse (donor mouse). Subsequent implantation of tumor pieces from donor mice onto recipient mice was performed based on a previously established protocol [16]. The implantation and growth of any biopsy tissue did not exceed five *in vivo* passages in order to minimize heterogeneity of tumor growth. PDX tumors were allowed to grow to approximately 120 mm^3 prior to initiation of treatment.

HT (*i.e.* 42.5 ± 0.5 °C) was applied locally at the tumor volume using a custom-designed laser-based heating setup [16,28]. For mice receiving HT treatment, tumors were preheated to 42.5 °C for 5 min prior to injection of drug therapy and heated for an additional 20 min post-injection at each treatment dose [16].