Bioelectrochemistry 119 (2018) 161–171

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Comparable effectiveness and immunomodulatory actions of oxaliplatin and cisplatin in electrochemotherapy of murine melanoma

Katja Ursic [a], Spela Kos [a], Urska Kamensek [a], Maja Cemazar [a,b], Janez Scancar [c], Simon Bucek [d], Simona Kranjc [a], Barbara Staresinic [a], Gregor Sersa [a,e,*]

[a] Institute of Oncology Ljubljana, Department of Experimental Oncology, Zaloska cesta 2, 1000 Ljubljana, Slovenia
[b] University of Primorska, Faculty of Health Sciences, Polje 42, 6310 Izola, Slovenia
[c] Jozef Stefan Institute, Department of Environmental Sciences, Jamova 39, 1000 Ljubljana, Slovenia
[d] Institute of Oncology Ljubljana, Department of Cytopathology, Zaloska cesta 2, 1000 Ljubljana, Slovenia
[e] University of Ljubljana, Faculty of Health Sciences, Zdravstvena pot 5, 1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 25 April 2017
Received in revised form 17 August 2017
Accepted 18 September 2017
Available online 03 October 2017

Keywords:
Oxaliplatin
Cisplatin
Electrochemotherapy
Melanoma
Immunogenic cell death
Immune response

ABSTRACT

Interest in platinum-based chemotherapeutics such as oxaliplatin (OXA) and cisplatin (CDDP) has been reinvigorated by their newly described impacts on tumor-specific immune responses. In addition to CDDP, OXA is frequently used to treat cancers. Based on the characteristics of OXA, which are similar to those of CDDP, and the presumably more pronounced immunomodulatory effect of OXA, OXA is a candidate for electrochemotherapy (ECT). We compared the effectiveness of intratumoral ECT with OXA to that of ECT with CDDP in murine B16F10 melanoma to determine the equieffective dose. Special attention was given to the elicitation of immunogenic cell death and local immune response. Based on the *in vitro* and *in vivo* results pertaining to effectiveness and drug uptake in cells and tumors, ECT with OXA is as effective as ECT with CDDP when the OXA dose is increased 1.6-fold. Exposure of melanoma cells to ECT induces immunogenic cell death when either OXA or CDDP is used, which correlates with a comparable increase in lymphocyte infiltration into tumors after ECT with either OXA or CDDP. Based on these results, OXA is a valid platinum-based drug for use with ECT, and the effectiveness of ECT with OXA is comparable to that of the well-established ECT with CDDP. Furthermore, both drugs display equal and specific immune responses following ECT.

1. Introduction

Platinum-based chemotherapeutic drugs are widely used in cancer treatment [1,2]. After the establishment of cisplatin (CDDP), other analogues such as oxaliplatin (OXA) and carboplatin, were introduced [1]. CDDD is used to treat ovarian, testicular, bladder, colorectal, lung as well as head and neck cancers [3]. In addition to metastatic colorectal cancer, OXA has also been used for treatment of cisplatin-resistant cancers, including stomach, pancreas, ovary, breast and lung cancers [1,4].

Mechanistically, DNA is the most important target of all platinum-based drugs [5–7]. Both, OXA and CDDP bind to DNA and form platinum-DNA adducts, though OXA does so to a lower extent [8,9]. Furthermore, aside from their cytotoxic effects *via* covalent binding to DNA, both OXA and CDDP act indirectly through protein and RNA binding and *via* immunomodulatory effects [7,10–12]. Specifically, after chemotherapy with OXA or CDDP, many of the damage-associated molecular patterns (DAMPs) are secreted, released or exposed [10,13]. It was recently reported that chemotherapy with CDDP result in the release of at least 19 DAMPs [14]. Moreover, the availability of at least three specific DAMPs, namely, calreticulin, adenosine triphosphate (ATP) and high mobility group box-1 protein (HMGB1), shifts the non-immunogenic mechanism of cell death in immunogenic cell death, which enables priming of an immune response against antigens released from dying cells [15,16]. It has been shown that, in contrast to OXA, CDDP does not elicit immunogenic cell death in several colon cancer cell lines [17]. Specifically, an inability of CDDP to induce full-blown endoplasmic reticulum stress prevents calreticulin redistribution from the endoplasmic reticulum to the plasma membrane [17,18]. As stated above, calreticulin exposure has been shown to be independent of the DNA-binding effects of OXA since treated cytoplasts exposed calreticulin successfully [15,19].

To increase platinum-based drug delivery across the cell membrane and consequent DNA binding and cytotoxicity, different drug delivery approaches have been tested previously. These approaches include Lipoxal™ and Lipoplatin™, which are liposomal vesicles loaded with OXA or CDDP [20–22]. Electroporation, where electric pulses are applied to increase the permeability of the cell membrane, is one of many approaches [23]. Electrochemotherapy (ECT), the delivery of drugs by

* Corresponding author at: Institute of Oncology Ljubljana, Department of Experimental Oncology, Zaloska cesta 2, 1000 Ljubljana, Slovenia.
E-mail address: gsersa@onko-i.si (G. Sersa).

https://doi.org/10.1016/j.bioelechem.2017.09.009
1567-5394/

electroporation, has already entered routine clinical use for delivery of CDDP [24] and bleomycin [25]. *In vitro*, it has been shown that the cytotoxicity of naive and CDDP-resistant cells is increased by exposure of cells to electroporation. Furthermore, the exposure of both cell lines to electroporation at an equitoxic concentration (IC_{50}) of CDDP increases platinum binding to DNA to the same level [26]. There is a direct correlation between the DNA platinum adduct levels and treatment efficacy [27,28]. *In vivo*, in preclinical studies, ECT with intratumoral (*i.t.*) CDDP was more effective than ECT with intravenous CDDP [29]. The first clinical study showed that ECT using *i.t.* CDDP administration is a highly effective approach for treating cutaneous malignant melanoma nodules [30]. Moreover, the ESOPE study, demonstrated that ECT with CDDP is an easy, highly effective, safe and cost-effective approach for treating the cutaneous and subcutaneous tumor nodules of different malignancies [31]. Electrochemotherapy with *i.t.* CDDP administration has been defined as a "Standard operating procedures" in clinics [32], and it is also used in veterinary oncology [33].

While ECT with CDDP is already a well-established treatment, OXA has not been tested for use with ECT. Due to its induction of immunogenic cell death [17], it could be potentially very effective in combined treatments with immunostimulators or with immune checkpoint inhibitors [34–37]. In addition, bleomycin is also known to induce immunogenic cell death [38] and would therefore strongly qualify for the combined treatment with modulation of the immune response. Moreover, CDDP, in contrast to OXA, does not induce immunogenic cell death [17]. However, it is not known whether combining CDDP with electroporation leads to immunogenic cell death comparable to immunogenic cell death after treatment with OXA alone. It is also not known whether immunogenic cell death is enhanced after ECT with OXA in comparison to chemotherapy with OXA.

Therefore, with the aim to increase the armamentarium of drugs for ECT, OXA was compared to the already established CDDP approach in the treatment of melanoma, and the dose modifying factor for equieffectiveness was determined. To explain the background mechanism, the platinum content in cells, the platinum level bound to DNA and the extent of calreticulin exposure on the surfaces of the treated cells were measured. Finally, we investigated how the choice of chemotherapeutic drug modifies the tumor microenvironment, which was indicated by the infiltration of tumors by granzyme B positive cells.

2. Material and methods

2.1. Tumor cells and animals

Murine B16F10 melanoma cells with high metastatic potential (American Type Culture Collection, Manassas, VA, USA) and B16F10 melanoma cells stably transfected with tdTomato fluorescent marker (B16F10 tdTomato; a kind gift from Muriel Golzio, Institute of Pharmacology and Structural Biology, Toulouse, France) were cultured as monolayers at 37 °C in a 5% CO_2 humidified incubator in Advance Minimum Essential Medium (AMEM, Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 5% fetal bovine serum (Gibco), 10 mM L-glutamine (GlutaMAX, Gibco), 100 U/mL penicillin (Grünenthal, Aachen, Germany) and 50 mg/mL gentamicin (Krka, Novo mesto, Slovenia).

Six- to eight-week-old female C57Bl/6 mice (Envigo Laboratories, Udine, Italy) and 12-week-old SKH1 hairless mice (Charles Rivers, Wilmington, MA, USA) were used. All procedures were performed in compliance with the guidelines for animal experiments of the EU Directives, the permission of the Ministry of Agriculture and the Environment of the Republic of Slovenia (Permission No. U34401-1/2015/16), which was provided based on the approval of the National Ethics Committee for Experiments on Laboratory Animals. Mice were kept in a specific pathogen-free environment with 12-hour light/dark cycles at 20–24 °C with 55% ± 10% relative humidity and given food and water *ad libitum*.

2.2. Drug formulation

OXA (Oxaliplatin Teva, 5 mg/mL, Castleford, United Kingdom) and CDDP (Cisplatina Kabi, 1 mg/mL, Fresenius Kabi AG, Bad Homburg, Germany) were diluted in AMEM (for *in vitro* experiments) or in a physiological solution (for *in vivo* experiments) to obtain appropriate molar concentrations. For each experiment, a fresh solution was prepared and kept in the dark before use.

2.3. In vitro electroporation protocol and clonogenic assay

Clonogenic assays were performed to determine the cellular reproductive potential after electroporation with OXA or CDDP. B16F10 melanoma cells were trypsinized and washed with ice cold electroporation buffer (125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2 \times 6H_2O$), and a cell suspension in electroporation buffer (2.2×10^7/mL) was prepared. OXA or CDDP within a range of drug concentrations (5 μM–150 μM) or cell growth medium alone (control) were added to the marked cell suspension (1×10^6/100 μL). Half of the suspension was used as a control, and the other half was electroporated. Electric pulses (parallel stainless-steel plate electrodes with a 2-mm distance between them, 8 square wave pulses at a frequency of 1 Hz, a voltage-to-distance ratio of 1300 V/cm and a duration of 100 μs) were applied with the GT-01 electric pulse Thereafter the cells were placed in a well of 24-well low attachment plate and incubated for 5 min at room temperature. After incubation the cells were resuspended in 2 mL of medium. For clonogenic assays, cells (200–3000, depending on drug concentration) were seeded in 6-cm Petri dishes in 4 mL of medium. After six days, formed colonies were stained with crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) and counted. The results are presented as the surviving fraction, which was calculated from the plating efficiency as described previously [39].

2.4. Determination of apoptotic and necrotic cell death in vitro

The type of cell death was determined 24 h after electroporation using a FITC Annexin V Apoptosis Detection Kit with 7-AAD (7-aminoactinomycin D, BioLegend, San Diego, CA) according to manufacturer's instructions. For excitation, a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA), with a 488-nm laser (air-cooled, 20 mW solid-state), was used, and for the detection of green and red fluorescence, 530-nm and 650-nm bandpass filters were used. Negative control (without electroporation and incubation with chemotherapeutic drug) was used to determine gating regions and discerning between apoptotic and necrotic cells.

2.5. Flow-cytometric analysis of surface-exposed calreticulin

Flow cytometry measurements were performed to evaluate calreticulin exposure on the plasma membrane in response to electroporation of B16F10 melanoma cells. Four hours after *in vitro* electroporation, cells were collected (1500 rpm, 5 min), washed once with ice-cold PBS and fixed in 0.25% paraformaldehyde (Alfa Aesar, Karlsruhe, Germany) in PBS for 5 min. After washing again with cold PBS, cells were incubated for 25 min at 4 °C with primary anti-calreticulin antibodies (Abcam), diluted in ice-cold blocking buffer (1% BSA in PBS), followed by washing twice with cold PBS and incubation with secondary Goat anti-Rabbit IgG H&L antibodies (Alexa Fluor® 488, Abcam), diluted in ice-cold blocking buffer (1% BSA in PBS), for 25 min at 4 °C. Cells were then washed with cold PBS twice and resuspended in 300 μL of cold PBS. Immediately after the addition of 4 μL of Propidium Iodide (PI, Sigma-Aldrich) measurements were performed. Propidium Iodide was used to distinguish between viable and dead cells. Samples were measured using a FACSCanto II flow cytometer (BD Biosciences) equipped with 2 lasers (488 and 633 nm), and the results were analyzed with the BD FACSDiva 8.0 software (BD Biosciences). At least 100,000