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O, O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl) propanoate dihydrochloride enhances influx of effective NK and NKT cells in murine breast cancer

O, O'-dietyl-(S,S)-etilendiamin-N,N'-di-2-(3-cikloheksil)propanoat dihidrohlorid povećava influks efektivnih NK i NKT ćelija u karcinomu dojke miša

Milena Jurišević*, Nikola Jagić, Nevena Gajović, Aleksandar Arsenijević*, Milan Jovanović, Marija Milovanović, Jelena Pantić, Ivan Jovanović, Tibor Sabo, Gordana D. Radosavljević, Nebojša Arsenijević*

1Equally contributed first author

University of Kragujevac, Faculty of Medical Sciences, *Center for Molecular Medicine and Stem Cell Research, 1Department of Pharmacy, 1Department of Radiology, Kragujevac, Serbia; Military Medical Academy, 1Department of Abdominal Surgery, Belgrade, Serbia; University of Defence, 1Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia; University of Belgrade, 1Faculty of Chemistry, Belgrade, Serbia

Abstract

Background/Aim. O, O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) has been found to possess promising cytotoxic activity against various tumor cell lines. Also, DE-EDCP reduces tumor progression by several mechanisms such as triggering tumor cell death and inhibition of cell proliferation. The aim of present study was to further evaluate antitumor activity of DE-EDCP by investigating effects on migratory potential of tumor cells and anti-tumor immune response. Methods. Migratory potential of DE-EDCP was evaluated by scratch wound assay. Female BALB/c mice were inoculated with 4T1 breast cancer cells and treatment with DE-EDCP started five days following orthotopic tumor implantation. The frequency and phenotype of tumor-infiltrating natural killer (NK) and natural killer T (NKT) cells were analyzed by flow cytometry. Results. DE-EDCP inhibited migratory potential of highly metastatic 4T1 cells.

Conclusion. DE-EDCP inhibits murine breast cancer progression through direct effects on tumor cells and by facilitating anti-tumor immunity. DE-EDCP enhances accumulation, promotes tumoricidal phenotype and maintains responsiveness of NK and NKT cells in 4T1 murine breast cancer.

Key words: breast neoplasms; carcinoma; mice, inbred balbc; antineoplastic agents.

Apstrakt

Uvod/Cilj. O, O'-dietil-(S,S)-etilendiamin-N,N'-di-2-(3-cikloheksil)propanoat dihidrohlorid (DE-EDCP) poseduje značajnu citotoksčku aktivnost na linije različitih tumorskih ćelija. DE-EDCP redukuje rast i metastaziranje karcinoma dojke tako što indukuje ćeljsku smrt i inhibira progresiju čelijskog ciklusa. U cilju dalje analize antitumorske aktivnosti DE-EDCP ispitana je njegov uticaj na migratori potencijal, kao i na antitumorski imunski odgovor. Metode. Uticaj DE-EDCP na mobilnost malignih ćelija ispitan je testom migracije (scratch wound assay). Model karcinoma dojke je indukovan ortotopskom transplanteacijom malignih ćelija 4T1 u singene ženke BALB/C miševa. Nakon pojave palpabil-
Ester derivatives of (S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl) propanoic acid were synthesized as ligands for ethylenediamine-based platinum complexes. These platinum(IV) complexes exhibit higher tumoricidal activity toward several cancer cell lines compared to cisplatin as a conventional chemotherapeutic drug. The cytotoxicity of platinum(IV) complexes could be at least partly related to their organic ligands with less of a relationship between the cytotoxic capacity and the alkyl side-chain length of these ligands. In line with this observation, it was further demonstrated that ligand O,O′-diethylyl-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl) propanoate dihydrochloride (DE-EDCP) alone exerted similar or even higher cytotoxic activity compared to cisplatin against various human and mouse cell lines. Taken together, it seems that DE-EDCP exerted highly potent cytotoxic activity against murine melanoma cells and human promyelocytic leukemia cells. Next, cytotoxicity of DE-EDCP was demonstrated on various leukemic cell lines. According to obtained IC_{50} values, human promyelocytic leukemia cells were highly sensitive to DE-EDCP. The cytotoxic effect of DE-EDCP against human leukemic-60 (HL-60) cells was accompanied with increased production of superoxide and depolarization of mitochondrial membrane. Results of this study indicated that DE-EDCP treatment caused caspase-independent apoptosis of HL-60 cells by presentation of phosphatidylserine on cell membrane and fragmentation of DNA.

Recently, we demonstrated that DE-EDCP attenuated murine breast cancer progression by facilitating apoptosis and inhibiting proliferation of tumor cells. DE-EDCP in cell line of murine breast cancer (4T1) tumor cells reduced expression of anti-apoptotic Bcl-2 while increased expression of pro-apoptotic Bax and caspase-3. Also, DE-EDCP treatment blocks cell cycle progression in 4T1 tumor cells by increasing expression of cyclin-dependent kinase inhibitors p16, p21 and p27 with subsequent decrease in the expression of cyclin D3 and Ki-67, and arresting 4T1 cells in G0/G1 phase of cell cycle. Further, DE-EDCP reduced the malignant potential of tumor cells by reducing expression of signal transducer and activator of transcription 3 (STAT3) and downstream regulated molecules, NANOG and SOX2 in 4T1 cells. Recent study also reported that DE-EDCP reduces melanoma growth mainly by inducing expression of key pro-apoptotic genes. Melanoma cells treated with DE-EDCP underwent caspase-dependent apoptosis as a result of mitochondrial dysfunction and increased accumulation of reactive oxygen species. In both murine breast cancer and melanoma models, DE-EDCP was well tolerated in vivo without obvious side effects.

Activity of natural killer (NK) cells, as well as natural killer T (NKT) cells, represents the major mechanism of innate immunity against tumors. NK cells lyse tumor cells without prior sensitization and represent the first line of defense against established tumors. NKT cells, by production of various immunoregulatory cytokines, link innate and adaptive immune response. It has been reported that NKT cell reduce tumor progression, mostly by enhancing cytotoxicity and interferon-gamma (IFN-γ) production of NK cells and CD8+ T cells.

The aim of this study was to further evaluate anti-tumor activity of DE-EDCP in 4T1 murine breast cancer model. We investigated the effects of DE-EDCP on migratory potential of tumor cells as well as modulation of anti-tumor immune response.

**Methods**

**Cell culture and reagents**

The cell line of murine breast cancer was purchased from American Type Culture Collection (ATCC, USA). 4T1 cells were grown in suspension in complete Dulbecco Modified Eagles Medium (DMEM) in a 5% CO2 incubator with standard conditions. Tumor cell suspension with > 90% viability was prepared using 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS, PAA Laboratories GmbH). In all in vitro and in vivo experiments only cell suspensions with > 95% viable cells were used. In order to determine the viability of tumor cells trypan blue was used.

The organic compound DE-EDCP, was prepared according previously described procedure. As a referent cytostatic, cisplatin (CDDP), cis-diaminedichloroplatinum(II)/cis-[PtCl2(NH3)2] (Sigma-Aldrich) was used.
Scratch wound assay

The wound healing assay was performed as previously reported. After 4T1 cells were seeded into 6-well plates, they were allowed to grow to about 90% confluence in present of complete medium. After 4T1 achieved appropriate confluence, a plastic tip was used to make a scratch on the cell monolayer. The wound area was washed three times with PBS and the 4T1 cells were incubated with DE-EDCP (15.63 µM) or cisplatin (CDDP) (15.63 µM) for 4 and 15 hours. The 4T1 cells migrated into the wound surface and the average distance of the migrating cells was observed using inverted microscopy. All data were analyzed from three independent experiments performed in triplicate using ImageJ software and the results are presented as the mean ± standard deviation (SD).

Animals

Female (8–12 weeks old) BALB/c mice were used in in vivo experiment. Experimental animals were equalized in weight and randomized in the experimental or control groups. The mice were housed in a temperature-controlled environment with a 12-hour light-dark cycle, fed ad libitum and observed daily. All experiments were approved by the University Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Animal model and drug treatment

BALB/c mice were inoculated with 3 × 10^4 4T1 tumor cells orthotopically into the fourth mammary fat pad. Pharmacology treatments started when tumors were palpable five days after implantation of 4T1 cells. Tumor bearing mice received intraperitoneal injection of either DE-EDCP (10 mg/kg/dose - five consecutive doses + two days pause + five consecutive doses; ten doses in total); CDDP (3 mg/kg/dose; three times per week; nine doses in total) or 0.9% NaCl. Mice were sacrificed on 18th day of the experiment.

Flow cytometric analyses of tumor-infiltrating NK and NKT cells

After three experimental groups of mice were sacrificed on 18th day of the experiment, primary tumor was isolated from mice and single cell suspensions of primary tumors were obtained by enzymatic digestion, as previously described. Fluorochrome-conjugated monoclonal antibodies specific for CD3 (145-2C11), CD49b (HMA2), CD178/FasL (MFL3), CD152/CTLA-4 (BNI3), PD-1 (PD1C1/922), KLRG-1 (2F1) or their respective isotype controls (BD Pharmingen, NJ/Invitrogen, Carlsbad, CA) were used. Expression of cell surface antigens was analyzed with Flow Cytometer (BD Biosciences, San Jose, CA) and the data were analyzed using FlowJo (Tree Star). Data are presented as means ± SD of two individual experiments, each carried out with six mice per experimental group.

DE-EDCP reduces migration of 4T1 cells

It is well known that cell migration is the first step in the invasive-metastatic cascade. We recently reported that DE-EDCP reduces murine breast cancer growth and metastasis. Herein, we add the effect of DE-EDCP treatment on 4T1 cell migration examined by wound healing assay using non-lethal concentration (15.63 µM) for 4 and 15 hours. Migration assay revealed that scratch wound area in wells with untreated 4T1 cells had a significant diminution (approximately 65%), while wound area of 4T1 cells treated with DE-EDCP was significantly wider in comparison to control cells 4 hours following treatment (p = 0.003; Figure 1). The same phenomenon was observed 15 hours after scratch (p = 0.019; Figure 1). In addition, significant effect of CDDP on the reduction of 4T1 cell migration was achieved after 15 hours (Figure 1).

DE-EDCP administration facilitates accumulation of NKT and NK cells within tumor microenvironment

We further analyzed the effect of DE-EDCP on local antitumor immune response. The obtained data revealed that DE-EDCP significantly increased the percentages of CD3/CD49^+ NKT cells in tumor tissue when compared to vehicle-treated mice (p = 0.03; Figure 2, left panel). Of note, the frequencies of NKT cells were increased in mice treated with CDDP, but it did not reach statistical significance (Figure 2, left panel). DE-EDCP also increased the percentages of tumor-infiltrating CD3/CD49^+ NK cells (p = 0.032; Figure 2, right panel). CDDP did not significantly affect accumulation of NKT cells in tumor tissue (Figure 2, middle panel). We did not reveal effect of DE-EDCP administration, or CDDP, on intratumoral accumulation of CD3^+CD49^+ T cells (Figure 2, right panel).

DE-EDCP affects functional phenotype of tumor-infiltrating NKT and NK cells

Further, we analyzed functional phenotype of tumor-infiltrating NKT and NK cells. Apart from CDDP, DE-EDCP did not affect the presence of tumoricidal NKT cells expressing FasL (Figure 3). However, DE-EDCP significantly decreased the presence of NKT cells expressing inhibitory markers such as CTLA-4, KLRG-1 and PD-1 in comparison with vehicle and CDDP treated mice (Figure 3).
In contrast to DE-EDCP, CDDP significantly increased the percentage of PD-1 positive NKT cells when compared to vehicle-treated mice (Figure 3).

Furthermore, mice treated with DE-EDCP, but not CDDP, exhibited significantly increased percentages of tumoricidal FasL⁺ NK cells compared to vehicle-treated mice (Figure 4). There were no significant differences in the expression of inhibitory KLRG-1, CTLA-4 and PD-1 among NK cells from both DE-EDCP and CDDP treated mice (Figure 4).

**Fig. 1** – Inhibitory effect of O,O’-diethyl-(S,S)-ethylenediamine-N,N’-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) on murine breast cancer cell migration.

The scratch wound assay of 4T1 cells treated with DE-EDCP (15.63 µM) or cisplatin (CDDP) (15.63 µM) for 4 hours and 15 hours. Representative images of wound closure in the control, CDDP and DE-EDCP treated cell line of murine breast cancer (4T1) cells. The images were captured three times at different areas and the results were analyzed by ImageJ software. Cell migration was quantified measuring the mean cell-free gap distance between the edges of the scratch area. Data are presented as mean of wound area ± standard deviation (SD). Mann-Whitney U test was performed and significant differences are reported (*p < 0.05).
Fig. 2 – O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) increases influx of natural killer T (NKT) and natural killer (NK) cells in tumor microenvironment. The graphs and representative sorting cells based on flow cytometry data (FACS) plots showing the percentages of NKT, NK and T cells derived from tumor tissue of vehicle-treated, cisplatin (CDDP)-treated and DE-EDCP-treated mice 18 days after cell line of murine breast cancer (4T1) cell inoculation. Data are presented as mean ± standard deviation (SD) of two individual experiments, each carried out with six mice per group. Statistical significance was tested by Mann-Whitney U test (*p < 0.05).

Fig. 3 – O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) affects the functional phenotype of natural killer T (NKT) cells in tumor tissue. The graphs and representative sorting cells based on flow cytometry data (FACS) plots show the percentage of Fas ligand (FasL+), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4+), killer cell lectin like receptor G1 (KLRG-1+) and programmed cell death protein-1 (PD-1+) NKT cells derived from tumor tissue of vehicle-treated, cisplatin (CDDP)-treated and DE-EDCP-treated mice 18 days after cell line of murine breast cancer (4T1) cell inoculation. Data are presented as means ± standard deviation (SD) of two individual experiments, each carried out with six mice per group. Statistical significance was tested by Mann-Whitney U test (*p < 0.05).
Fig. 4 – O,O’-diethyl-(S,S)-ethylenediamine-N,N’-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) affects the phenotype of natural killer (NK) cells in tumor tissue.

The graphs and representative flow cytometry data (FACS) plots show the percentage of fas ligand (FasL +), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 +), killer cell lectin like receptor G1 (KLRG-1 +) and programmed cell death protein-1 (PD-1 +) NK cells derived from tumor tissue of vehicle-treated, cisplatin (CDDP)-treated and DE-EDCP-treated mice 18 days after cell line of murine breast cancer (4T1) cell inoculation. Data are presented as means ± standard deviation (SD) of two individual experiments, each carried out with six mice per group. Statistical significance was tested by Mann-Whitney U test (*p < 0.05).

Discussion

Cell migration is a prerequisite for tumor invasion and metastasis 17, and can be a potential therapeutic target for tumor treatment. For this purpose we used 4T1 cells, known as cells with high metastatic potential 18. Herein, our results indicated that DE-EDCP effectively inhibits the migration of 4T1 cells as evaluated by wound healing assay (Figure 1). We previously reported that DE-EDCP decreased expression of signal transducer and activator of transcription 3 (STAT3) in 4T1 cells, as well as NANOG and SOX2 which are downstream targets of STAT3 signaling pathway 4. STAT3 has impact on cell invasion and motility 19. For instance, knockdown of STAT3 compromised the proliferation and migration of Michigan Cancer Foundation-7 (MCF7) human breast cancer cells 20. In addition, overexpression of NANOG promoted the migration and invasion of MCF7 cells 21. Similarly, NANOG regulated cell migration in ovarian cancer 22. Furthermore, SOX2 silencing has also been found to prevent migration of MDA-MB-231 human breast cancer cells 23. In agreement with these findings, it appears that DE-EDCP inhibits tumor cell migration via downregulation of STAT3, NANOG and SOX2 expression. Therefore, inhibition of 4T1 cell migration seems to be the additional beneficial effect of DE-EDCP on breast cancer progression.

In addition to obvious direct effects on tumor cells, we further hypothesized that DE-EDCP might influence tumor progression by modulating anti-tumor immune response. To the date, it was found that DE-EDCP inhibited production of IFN-γ and IL-17 by cells derived from spleen and lymph nodes of mice and rats 24. However, the effects of DE-EDCP on anti-tumor immune response are still unknown. In this study, we explored the effects of DE-EDCP on anti-tumor innate immunity in the weakly immunogenic and highly metastatic 4T1 murine mammary cancer model. We showed that DE-EDCP facilitated influx of CD3⁺CD49⁺ NKT cells and CD3⁺CD49⁻ NK cells in tumor microenvironment (Figure 2). CDDP treatment slightly increased influx of these
CD8+ T cells plays a nonessential role in 4T1 breast tumor 11, 35. The influence of DE-EDCP on the functional status of these cells, however the increment did not achieve statistical significance so we can only assume that CDDP antitumor effects in particular tumor model were achieved by some other mechanisms. NK cells are innate immune effector lymphocytes that play an important role in the protection against tumor. NK cells infiltrate solid tumors thus contributing to favorable prognosis in cancer patients 25. Apart from NK cells, activated NKT cells are involved in elimination of tumor cells either directly or indirectly by engagement of other immune cells 26–27. Furthermore, recently an association between numbers of tumor-infiltrating NK cells with better clinical outcome was found36. NKT cells react quickly to stimuli and have a remarkable capacity to produce an array of cytokines and chemokines in order to modulate both innate and adaptive immune response 29.

In addition to increased influx of NKT and NK cells in breast cancer tissue, the obtained data revealed that DE-EDCP affects functional phenotype of these cells. It is well-established that both cell types, in particular NK cells, directly eliminate target tumor cells by at least two mechanisms, producing perforins and granzymes as well as the engagement of cell death receptors. Cell death receptor Fas and its ligand FasL are important players in initiation of target cell apoptosis 30. Fas-FasL interaction induces receptors trimerization, activation of adaptor protein fas-associated protein with death domain (FADD) which results with activation of caspase-8 and consequent initiation of apoptosis 31. Our results revealed that DE-EDCP treated mice had significantly increased percentages of FasL+ NK cells, but not NKT cells, indicating their enhanced tumoricidal potential (Figures 3 and 4). These data are in line with our previously described results revealing that DE-EDCP treatment increased percentage of apoptotic (TUNEL+) tumor cells in breast cancer tissue 4. NKT cells directly eliminate CD1d-expressing tumor cells 32. 4T1 cells express minimal surface levels of CD1d 33. Therefore, there is low possibility that NK cells directly eliminate 4T1 cells. However, NK cells could produce IL-2 further stimulating NK cells to kill the NKT cell-resistant tumor cell targets 34. Teng et al. 33 showed that CD8+ T cells and IFN-γ are crucial for 4T1 tumor eradication. However, other studies revealed that antitumor activity based on cytotoxicity of CD8+ T cells plays a nonessential role in 4T1 breast tumor model 11, 35. Innate immunity cells, especially NK cells, occupy a central place in the control of growth and metastasis of weakly immunogenic mouse 4T1 breast tumor 11, 35. The influence of DE-EDCP on the functional status of NK cells in the tumor microenvironment indicates that the DE-EDCP effects on the innate immune response may be an additional anti-tumor mechanism of action. At this point, we may suppose that higher percentage of tumor-infiltrating NK cells and higher expression of FasL on NK cells after DE-EDCP treatment may lead to Fas-FasL interaction of tumor and NK cells and, consequently, cause tumor cell death.

Also, we can only assume that DE-EDCP might stimulate NKT cells in tumor microenvironment to produce various cytokines thus enhancing tumoricidal capacities of NK cells. This can be an additional mechanism of DE-EDCP-mediated diminishing of tumor progression.

Killer cell lectin-like receptor G1 (KLRG-1) is C-type lectin-like inhibitory receptor expressed mostly on NK cells, cytotoxic T cells and long-lived effector NKT cells 36, 37. KLRG-1 regulates homeostasis and maturation of NK cells 38. High KLRG-1 expression correlates with low proliferative capacity 38, 39 and increases apoptosis of NK cells 40. DE-EDCP significantly decreased percentage of NKT cells, but not NK cells, expressing inhibitory receptors KLRG-1 (Figures 3 and 4). The programmed cell death-1 receptor (PD-1) is immune checkpoint inhibitor expressed on the surface of immune effector cells, including T cells, NK and NKT cells 41–43. Marked increase in PD-1 expression after α-GalCer stimulation indicated NKT cell anergy 44, 45. Herein, we observed significantly decreased percentage of PD-1+ NKT cells following DE-EDCP treatment (Figure 3) thus contributing to NKT cell responsiveness. Next, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is another immune checkpoint molecule with crucial role in decline of immune response and maintaining immune homeostasis 46, 47. CTLA-4 is expressed on tumor-infiltrating NK cells in mice 48. The obtained data revealed that DE-EDCP also reduced the frequencies of CTLA-4+ NKT cells (Figure 3). Both PD-1 and CTLA-4 blockade, as well as their combination, have proven to be very effective in animal models of melanoma and some breast cancer models 49–52.

Conclusion

In addition to our previously published data regarding the beneficial effects of DE-EDCP on 4T1 breast cancer progression, here we add the evidences that DE-EDCP inhibits 4T1 cell migration and promotes anti-tumor immune response mediated by NK and NKT cells. DE-EDCP enhances accumulation, promotes tumoricidal phenotype and maintains responsiveness of NK and NKT cells in 4T1 murine breast cancer model.

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Declaration of interest

The authors declare that they have no competing interests.
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