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SHORT COMMUNICATION

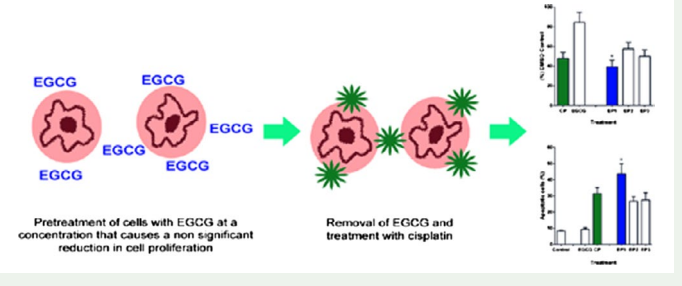
Modulation of cisplatin cytotoxic activity against leiomyosarcoma cells by epigallocatechin-3-gallate

Irida Th. Dhima[a], Dimitrios Peschos[a], Yannis V. Simos[a], Maria I. Gkiouli[b], Maria E. Palatianou[a,c], Vasilios N. Ragos[d], Vasiliki Kalfakakou[a], Angelos M. Evangelou[a] and Spyridon Ch. Karkabounas[a]

[a]Laboratory of Physiology, Faculty of Medicine, University of Ioannina, Ioannina, Greece; [b]Institute of Medical Engineering (IMETUM), Technical University of Munich, Munich, Germany; [c]Department of Internal Medicine, General Hospital of Nikaia Agios Panteleimon, Piraeus, Greece; [d]Maxillofacial Surgery, Faculty of Medicine, University of Ioannina, Ioannina, Greece

ABSTRACT

The aim of this study was to investigate the cytotoxic effect cisplatin in combination with epigallocatechin-3-gallate (EGCG) on leiomyosarcoma cells (LMS cells) in order to identify a less toxic but equally effective alternative. Assays for cell proliferation, colony formation efficiency, induction of apoptosis and cell cycle arrest were performed using the IC_{50} of cisplatin (8.6 μM) as a reference value and a concentration of EGCG (30 μM) that caused a non-significant reduction in cell proliferation. Pre-treatment of cells with EGCG for 24 h before the addition of cisplatin increased cytotoxicity up to 8.5% ($p < 0.05$) and the number of apoptotic cells by 40%. Epigallocatechin-3-gallate failed to alter S-phase cell cycle arrest induced by cisplatin and to modulate cisplatin effects on mitochondrial function. These results indicate that pre-treatment with EGCG could be used as an adjunctive therapy to maximise effectiveness of chemotherapy.



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1. Introduction

Cisplatin (cis Diamminedichloroplatinum) is one of the most potent platinum-based antitumour drugs used alone or in combination with chemotherapy regimens for the treatment

CONTACT Irida Th. Dhima iridadhima@gmail.com, isimos@cc.uoi.gr
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of broad spectrum of human tumours (Hannon 2007). It exerts high toxicity in a variety of cancer cells. Cisplatin activates mitogen-activated protein kinases, inducing apoptosis, and stimulating inflammation and fibrogenesis (Yao et al. 2007). Chemotherapy, despite its effectiveness, can affect some healthy, rapidly proliferating cells (Joy et al. 2008). Cisplatin is also characterised by its high toxicity, especially nephrotoxicity (Yao et al. 2007). An activated cisplatin molecule (once it enters the cell), which is strongly electrophilic, causes severe side effects induced by the oxidative stress (Cejas et al. 2004; Valko et al. 2006). Since plant derivatives are usually characterised by potent antioxidant properties, a new approach in cancer therapy could incorporate the combination of chemotherapeutic drugs with plant derivatives. Epigallocatechin-3-gallate has been implicated as an important bioactive molecule since it exhibits a wide range of biological effects: it may scavenge the free radical ions and increase the activity of antioxidant enzymes (Simos et al. 2011), inhibit cell proliferation, induce apoptosis in cancer cells, modulate cell cycle and interfere with intracellular signalling cascades like mitogen-activated protein kinases (MAPKs) (Chakrawarti et al. 2016). There are several studies investigating combinations of EGCG with various treatments against cancer e.g. EGCG in combination with 5-fluorouracil (Norwood et al. 2007; Yang et al. 2012), a purine nucleoside analogue (Nabhan et al. 2004) and with a DNA vaccine. (Kim et al. 2003). Recently, the U.S. Food and Drug Administration (2015) approved Yondelis (trabectedin) for the treatment of unresectable or metastatic liposarcoma and leiomyosarcoma. Most common side effects correlated with trabectedin are a reversible increase in transaminase levels, hematotoxicity (neutropenia and anaemia), hepatotoxicity and in some cases muscle tissue breakdown (rhabdomyolysis) (Recine et al. 2017). Ongoing clinical trials explore the combination of trabectedin with chemotherapy or new molecules. In this context and in order to find a less toxic but equally effective alternative we decided to evaluate the effects of EGCG on cisplatin cytotoxic activity against LMS cells.

2. Results and discussion

Analysis of data was determined by Student's t-test and one-way analysis of variance (ANOVA) (significance level was set at $p < 0.05$) (SPSS 16.0). The power curve fit model was used for curve fitting (GraphPad Prism 6 Software). The IC_{50} value of cisplatin was 8.6 ± 1.1 μM whereas the maximum concentration of EGCG that causes a non-significant reduction in cell proliferation was 30 μM (Figure S1A and S1B). The experimental protocols (EP) 1, 2 and 3 were then conducted (EP1: addition of EGCG 24 h before medium removal and the addition of cisplatin with fresh medium; EP2: simultaneous addition of cisplatin and EGCG; EP3: addition of EGCG 24 h after the addition of cisplatin). The results showed that pre-incubation of cells with EGCG (EP1) leads to an increase of cytotoxicity up to 8.5% ($p < 0.05$). In order for cisplatin to achieve similar cytotoxicity, the corresponding concentration should be raised to 19.4 μM, a 2.25-fold increase. Thus, a reduction of 56% in the required cisplatin concentration was achieved by pre-treatment with EGCG (Figure S1C). EGCG at concentrations lower than 100 μM enhanced MRC-5 cell proliferation (Figure S1B). Moreover, pre-treatment with EGCG (as EP1 in LMS cells) protected MRC-5 from the cytotoxic activity of cisplatin (Figure S1D). Simultaneous incubation of cells with EGCG and cisplatin (EP2), as well as the addition of EGCG subsequent to cisplatin (EP3) did not significantly alter the activity of cisplatin (Figure S2). Cisplatin increased the number of cells undergoing apoptosis, while EGCG itself had no effect. Pre-incubation of cells with EGCG (EP1) showed a significant increase of apoptotic

cells compared to simultaneous incubation of cells with EGCG and cisplatin (EP2), as well as the addition of EGCG subsequent to cisplatin (EP3). In comparison to cisplatin alone, EP1 raised the apoptotic cell number by 40% ($p < 0.05$). EP2 and EP3 had no significant effect on apoptotic cell death caused by cisplatin (Figure S3). At doses over 2 μM of cisplatin, LMS cells completely lost the ability to form colonies. One micromolar (1 μM) of cisplatin caused a significant reduction (58%, $p < 0.05$) in colony forming capacity. Incubation of cells only with EGCG (30 μM) led to a decreased number of colonies (20% less than untreated cells). Pre-incubation of cells with EGCG for 24 h and addition of 1 μM of cisplatin for 48 h, failed to enhance cisplatin inhibitory activity (Figure S4). Accumulation of cells in S-phase after treatment with cisplatin indicated that tumour cells had entered S-phase cell cycle arrest. Pre-treatment with EGCG (EP1) slightly reduced the number of cells in S-phase (from 69.8 ± 3.2% to 65.9 ± 2.8%). Moreover, the percentage of cells in G2/M phase decreased from 18.3 ± 1.9% to 9.9 ± 1.1% ($p < 0.05$) (Table S1 and Figure S5). The treatment of LMS cells with IC_{50} values of cisplatin presented a significant damage (more than 50%) in mitochondria of cells similar to that caused by oligomycin. Cells treaded only with EGCG lost almost 10% of mitochondrial activity. The EP1 failed to modulate cisplatin effects on mitochondrial function (Figure S6).

Cisplatin is an anticancer, antineoplasmatic chemotherapeutic drug used in the treatment of various types of cancer (Yao et al. 2007). Treatment with cisplatin induces two different modes of cell death necrosis and apoptosis. The DNA damage induced by cisplatin is characterised by the cell cycle arrest; inhibition of transcription and initiation of apoptosis. Excessive DNA damage induces hyperactivation of poly(ADP-ribose)polymerase, that can cause depletion of NAD^+/ATP. If ATP depletion reaches lethal-inducing levels necrotic cell death occurs (Wang and Lippard 2005). Mayr et al. 2015 studied the effect of EGCG alone and in combination with cisplatin on the viability of biliary tract cancer (BTC) cell line. Epigallocatechin-3-gallate in combination with cisplatin reduced the cell viability of BTC cell line in a concentration-dependent manner. The authors also investigated the effect of EGCG treatment on the expression of cell cycle and apoptosis-related genes. They concluded that EGCG reduced the expression of ccna2, ccnb1, ccnd1 and e2f1, but not of ccne1 in TFK-1 cells (cholangiocarcinoma) and enhanced level of dr5 and p21 gene expressions (Mayr et al. 2015). In a recent study, administration of EGCG after sunitinib treatment showed suppression of the MAPK signalling induced by sunitinib in human cancer cells (Zhou et al. 2016). Epigallocatechin-3-gallate in combination with 4-OHT (4-hydroxytamoxifen, used to treat oestrogen receptor-positive breast cancer) present synergistic cytotoxicity in MDA-MB-231 cells, greater than EGCG or 4-OHT alone (Chisholm et al. 2004). Also in another study, it is shown that epigallocatechin-3-gallate reduced the amount of cisplatin needed to accomplish growth inhibition in SKOV3, CAOV3 and C200 cells, the latter being a cell line that presents strong resistance to cisplatin (Chan et al. 2006). The authors suggested that EGCG may accentuate oxidative stress to inhibit growth of ovarian cancer cells and sensitise them to cisplatin. Wang et al. 2015 offered another possible mechanism according to which EGCG enhances copper transporter 1 (CTR1, a transporter that increases the cellular uptake and sensitivity of cisplatin) mRNA and protein expression in ovarian cancer cell lines SKOV3 and OVCAR3 (Wang et al. 2015). Epigallocatechin-3-gallate also enhances the ability of cisplatin to promote apoptosis in the prostate cancer cell line PC3 by promoting the expression of the pro-apoptotic splice isoform of caspase 9 (Hagen et al. 2013). Recently, the role of the 67 kDa laminin receptor (67LR) was acknowledged in the development of colon cancer multidrug resistance (Lu et al. 2016). The 67LR when overexpressed acts as a death receptor

(Kumazoe et al. 2013). This cell-surface receptor mediates the anti-cancer action of EGCG (Tachibana et al. 2004) although it has been shown that the enhancement of apoptosis and cancer cell death in combination treatment with EGCG can be independent of 67LR (Kondo et al. 2013). According to our results pre-treatment of cells with EGCG (EP1) increased susceptibility to cisplatin and decreased cell viability, enhanced cisplatin apoptotic effect, failed to alter S-phase cell cycle arrest induced by cisplatin, increased the percentage of cells in sub-G1 indicating apoptosis and failed to modulate cisplatin effects on mitochondrial function. It is of great importance to remember that the dose of EGCG used was the maximum concentration that causes a non-significant reduction in cell proliferation and thus, did not increase apoptotic cell death itself. Although treatment of cancer cells with EGCG enhances apoptosis (Singh et al. 2011) this was not the case in our protocol. Epigallocatechin-3-gallate exhibits a pro-oxidant effect at concentrations higher than 60 μM, whilst at low concentrations (2–30 μM) EGCG has the ability to scavenge reactive oxygen species (ROS) and predominates over its reducing power (Tian et al. 2007). Thus, any effects observed for the EGCG alone or in combination with cisplatin (the three experimental protocols) could not be attributed to the generation of free radicals by EGCG. The effectiveness of EP1 (over EP2 and EP3) indicated that the modulation of specific biological mechanisms was achieved by EGCG alone prior to the addition of cisplatin. Future experiments could reveal whether pre-treatment of cancer cells with EGCG activated a sufficient number of receptors enabling cisplatin to exert a greater apoptotic effect.

3. Conclusion

Only pre-treatment with EGCG sensitised LMS cells to cisplatin since the other two treatment schemes (simultaneous and post-treatment with EGCG) failed to exhibit an additive/synergistic effect. Further experiments are required in order to clarify whether EGCG could be a future adjunctive treatment of cisplatin, by enhancing its activity as well as reducing the side effects.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Yannis V. Simos http://orcid.org/0000-0003-1764-8906

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