Control of Neutrophil Endothelial Transmigration by EC-18 in Chemotherapy-Induced Neutropenia

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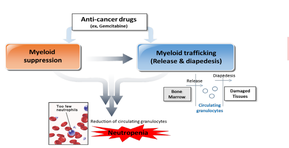
Abstract

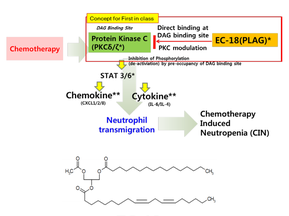
Chemotherapy-induced neutropenia (CIN) is a common side-effect that often compromises chemotherapy resulting in dose reduction and/or delayed treatment. Chemotherapy-induced myeloid suppression is prevalent mechanism to describe CIN. However, diapedesis of neutrophils from the blood is also a possible mechanism of CIN. Cytokines, chemokines and damage- (or danger-) associated molecular pattern molecules (DAMPs) are produced from the damaged tissues by chemotherapy, which accelerates neutrophil transmigration. EC-18 (1-palmityoyl-2-linoleoyl-3-acetyl-rac-glycerol) is a synthetic monoacetyl-diglyceride (MW=635) currently under development as an oral agent to treat and potentially prevent CIN. Here, we showed that neutrophil transmigration was induced in CIN model and the efficacy and potential mechanism of EC-18 have been evaluated in mice and various cell models. Gemcitabine (50mg/kg), cyclophosphamide (100mg/kg) or tamoxifen (50mg/kg) was administrated daily for 3 days in mice (C57BL/6), followed by their oral administration of EC-18 (50mg/kg) for 21 days, and blood neutrophils were analyzed by complete blood count (CBC) and fluorescence-activated cell sorting (FACS). All three chemotherapeutic agents significantly reduced absolute neutrophil count (ANC) in the blood; 40-4% decrease in gemcitabine, 19.8% in cyclophosphamide, and 21.8% in tamoxifen, which were recovered by the co-administration of EC-18. Microarray analysis of the lymph nodes from gemcitabine-treated mice revealed that the expression of DAMP molecules, S100A8 and S100A9, was induced by the drug and decreased by the co-treatment of EC-18. Using immune cell lines, the expression of neutrophil transmigratory molecules, such as C3 anaphylatoxin, IL-6 and IL-8, was shown to be increased by gemcitabine treatment, and EC-18 blocked the expression of those cytokines in dose-dependent manners. Western blot analysis showed that EC-18 regulated STAT3 and STAT6 signaling pathways to modulate the expression of DAMPs, C3, IL-6, and IL-8. The decrease of C3 and IL-6 levels by EC-18 treatment was also verified in PBMCs from normal adults. In a pilot study, orally administrated EC-18 (1,000mg/day) alleviated neutropenic phenotypes in patients with pancreatic cancer who had been treated with gemcitabine (1,000mg/m2) and erlobtinib (100mg daily). As a supportive care agent, EC-18 can maximize the antitumor efficacy of myelo-suppressive chemotherapeutic agents by enabling the treatment to continue or even higher doses to be administered.

Introduction

- Chemotherapy causes side-effect such as neutropenia, the decrease of absolute neutrophil count in the blood [1].
- S100A8 and S100A9 are well known damage- (or danger-) associated molecular pattern molecules (DAMPs) inducing neutrophil transmigration to inflammatory sites [2].
- C3 anaphylatoxin, IL-6, and IL-8 are well known neutrophil transmigratory molecules. C3 anaphylatoxin induces the expression of P-selectin, I-CAM1 or E-selectin in endothelial cells [3]. IL-6 and IL-8 are involved in neutrophil chemotaxis [4].
- EC-18 is a synthetic monoacetyl-diacylglyceride, and an effective regulator of TH2 immunity, allergic asthma, and rheumatoid arthritis [5].



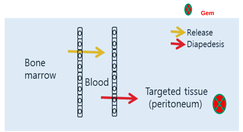
Mimetic diagram of chemotherapy induced neutropenia



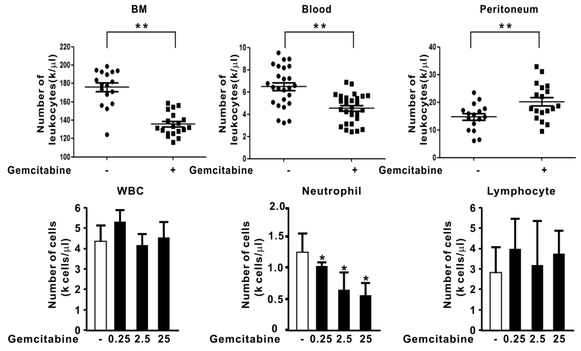
EC-18

Results

1. Gemcitabine induced leukocyte migration.

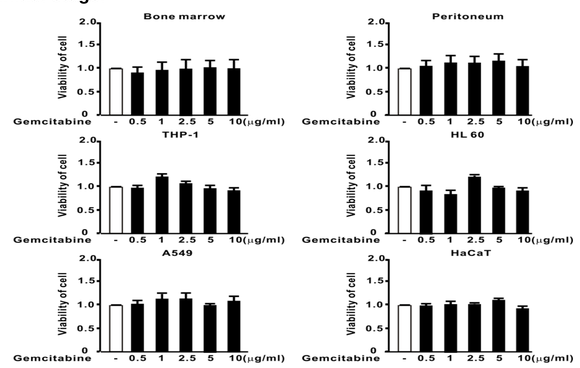


Leukocyte migration was observed in the gemcitabine treated mice after 15hr. Balb/c mice (Six week old, female) were administered gemcitabine (2.5 mg/kg) for 15 hr and then leukocytes in bone marrow, blood and peritoneum were evaluated by CBC analysis. * * $p < 0.001$ vs. no gemcitabine treated



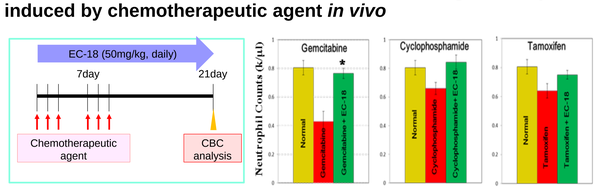
Gemcitabine induced neutropenia through neutrophil transmigration. 15 h after gemcitabine (0, 0.25, 2.5, or 25 mg/kg) treatment by intraperitoneal injection(IP), Neutrophils (but not lymphocytes) decreased with dose dependent manner. * $p < 0.05$ vs. no gemcitabine treated

2. Gemcitabine did not induce cytotoxicity (myelosuppression) at initial stage.

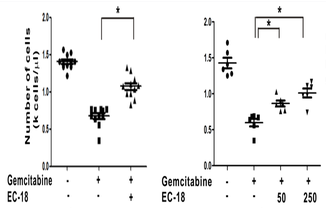


Cytotoxicity of gemcitabine in the primary cells and cell lines. Cytotoxicity was evaluated by Ez-cytox. Primary cells (BM and peritoneum) or tumor cells (THP-1, HL60, A549, and HaCaT) were cultured in 96-well plates with 5 x 10^3 cells/well and incubated for 15hr with serially diluted gemcitabine.

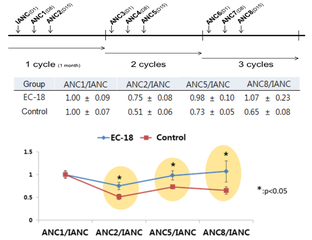
3. EC-18 inhibited the decrease of circulating neutrophils induced by chemotherapeutic agent *in vivo*



Balb/c mice (six week old, female) received daily dose of gemcitabine (50 mg/kg), cyclophosphamide (100 mg/kg), or tamoxifen (50 mg/kg) for 3 days by intraperitoneal injection followed by oral administration of EC-18 (50mg/kg) for 21 days. Blood neutrophils were analyzed by complete blood count (CBC) showing the recovery of blood neutrophils by EC-18. * $p < 0.05$ vs. gemcitabine only.



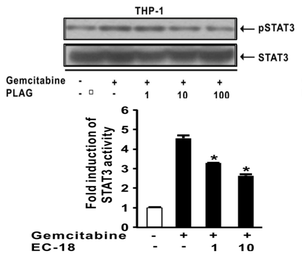
Balb/c mice (six week old, female) received 2.5 mg/kg of gemcitabine (IP) w/ or w/o EC-18 (PO). 15 h after injection, the total number of neutrophils in blood were counted by CBC analysis. * $p < 0.05$ vs. gemcitabine only.



Group	ANC1/IANC	ANC2/IANC	ANC5/IANC	ANC8/IANC
EC-18	1.00 ± 0.09	0.75 ± 0.08	0.98 ± 0.10	1.07 ± 0.23
Control	1.00 ± 0.07	0.51 ± 0.06	0.73 ± 0.05	0.65 ± 0.08

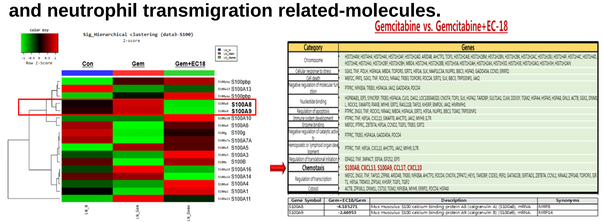
In a pilot study, EC-18 alleviated neutropenic phenotypes in pancreatic cancer patients treated with gemcitabine (1,000 mg/m^2) and erlotinib (100 mg/day). ANC, absolute neutrophil count; IANC, baseline neutrophil count prior to chemotherapy; ANC1, ANC obtained just prior to second gemcitabine injection; ANC2, ANC obtained just prior to third gemcitabine injection; ANC5, ANC obtained just prior to sixth gemcitabine injection; ANC 8, ANC obtained just prior to ninth gemcitabine injection. * $p < 0.05$ vs. chemotherapeutic agents only.

4. EC-18 regulated gemcitabine-induced STAT3 and STAT6 activation.

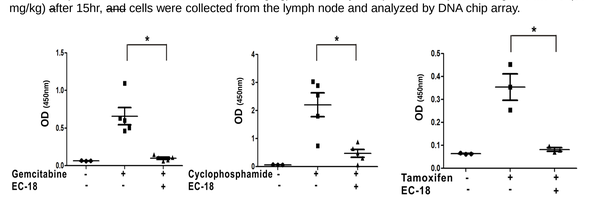


THP-1 cells was pretreated with 1, 10, and 100 μg/ml of EC-18 for 1 h and exposed to 10 μg/ml of gemcitabine for 30 min. The phosphorylation of STAT3 and STAT6 were analyzed by Western blotting (upper). RAW264.7 cells were transfected with STAT3 or STAT6 reporter construct, and treated with EC-18 (1 or 10 μg/ml) and gemcitabine (1 μg/ml) for 24 h. Transient expression of the reporter gene was quantified by luciferase assay (lower). * $p < 0.05$ vs. gemcitabine only.

5. EC-18 down-regulated the expression of DAMPs (S100A8, A9) and neutrophil transmigration related-molecules.

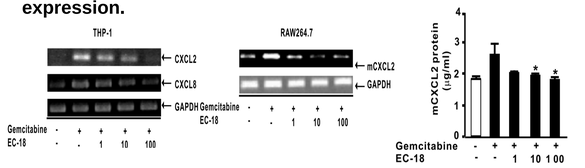


Gemcitabine-induced DAMPs (S100A8 and S100A9) were decreased by EC-18. Balb/c mice (six week old, female) received oral dose of EC-18 (50 mg/kg) followed by intraperitoneal injection of gemcitabine (50 mg/kg) after 15hr, ~~and~~ cells were collected from the lymph node and analyzed by DNA chip array.



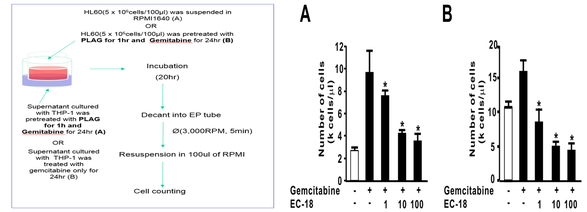
Chemotherapeutic agent-induced C3a was decreased by EC-18. Balb/c mice (six week old, female) received daily doses of gemcitabine (50 mg/kg), cyclophosphamide (100 mg/kg) or tamoxifen (50 mg/kg) for 3 days by intraperitoneal injection. Mice also received oral administrations of EC-18 (50mg/kg) for 21 days consecutively. Secreted C3a level was analyzed by ELISA from serum. * $p < 0.05$ vs. gemcitabine only.

6. EC-18 decreased gemcitabine-induced CXCL2 and CXCL8 expression.



THP-1 or RAW264.7 cell were pretreated with 1, 10, and 100 μg/ml of EC-18 for 1 h and exposed to 10ug/ml of gemcitabine for 4 h (RT-PCR) or 24 h (ELISA). RT-PCR analysis showed the expression of CXCL chemokines were reduced by EC-18 (left). The supernatants from RAW264.7 cells were analyzed by ELISA, and the production level of CXCL2(right) was reduced by EC-18. * $p < 0.05$ vs. gemcitabine only.

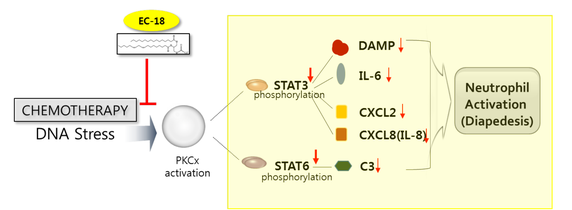
7. Neutrophil transmigration was reduced by EC-18 in vitro.



(A) THP-1 cells were treated with gemcitabine (10 μg/ml) and EC-18 (1, 10, and 100 μg/ml), and the culture supernatant was used to induce the transmigration of HL-60 cells. (B) HL-60 cells were pre-treated with gemcitabine (10 μg/ml) and EC-18 (1, 10, and 100 μg/ml), and the transmigration was analyzed using culture supernatant from THP-1 cells treated with gemcitabine (10 μg/ml) for 24 h. * $p < 0.05$ vs. Gemcitabine only.

Conclusion

- Number of circulating neutrophils decreased (i.e., neutropenia) significantly when treated with chemotherapeutic agents in vivo. Co-treatment with EC-18 maintained the number of circulating neutrophils to normal level.
- DAMPs (S100A8 and S100A9) and neutrophil transmigration-associated molecules (C3 anaphylatoxin, IL-6, and IL-8) were induced by chemotherapeutic agents and reversed by EC-18.
- EC-18 down-regulated DAMPs and neutrophil transmigration-associated molecules through the inhibition of STAT3 and STAT6 phosphorylation.
- EC-18 may prevent and/or treat neutropenia by inhibiting the transmigration of neutrophils in chemotherapy-treated patient.



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