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The ErbB1-Dependent Effect of Recombinant Epidermal Growth Factor (rEGF) Against Lapatinib-Induced Cytotoxicity in Caco-2 Human Intestinal Cells

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ABSTRACT

Lapatinib, an orally administered dual ErbB1 and ErbB2 tyrosine kinase inhibitor, is an FDA-approved treatment for ErbB2-positive breast tumour. However, diarrhoea is one of the major drawbacks for this treatment. Incidence of lapatinib-induced diarrhoea (LID) has been reported as high as 58-78% of treated patients, with 23.3-25% grade 3-4 cases. Although diarrhoea seems tolerable, prolonged diarrhoea can cause malnutrition, hospitalization and interfere with cancer treatment. Thus, diarrhoea can compromise the quality of life of patients with cancer. ErbB1 is widely expressed in the intestine which functions to maintain homeostasis. Thus, ErbB1 is hypothesised to be involved in LID mechanism through its inhibition by lapatinib in the intestine. The investigation aims to determine the cytotoxicity effect of lapatinib on Caco-2, a human colon adenocarcinoma cell line, and to determine whether recombinant epidermal growth factor (rEGF) could counteract lapatinib action. The experiments were conducted using water-soluble tetrazolium salt (WST-1) cell viability assay. Caco-2 was selected as an in vitro model as it is able to differentiate into enterocytes-like phenotype, hence reflecting small intestinal epithelium. Caco-2 was treated with a range of concentrations of lapatinib (0-100μM) and incubated at 24, 48, 72 and 96 hours. Lapatinib showed different inhibitory concentrations (IC_{50}) at different time-points, with no IC_{50} observed at 24 hours. Recorded IC_{50} values are 28μM ±12.81, 29μM ±2.51 and 14μM ±1.64 at 48, 72 and 96 hours, respectively. The median half maximal IC_{50} of lapatinib; 28.00μM ±2.51 over 48-96 hours was treated on Caco-2 in combination with rEGF (150-1000nM). rEGF was able to promote 35.5% *cell proliferation in lapatinib-treated cells at 325nM±5.18. Overall, rEGF counteracts lapatinib-induced cytotoxicity on the intestinal cells which proves lapatinib interaction with ErbB1 that leads to LID. However further investigations are required.*

Keywords: *Caco-2; Cell viability assay; ErbB1; Lapatinib, Recombinant epidermal growth factor (rEGF)*

1. INTRODUCTION

Lapatinib is a potent ErbB1 and ErbB2 tyrosine kinase inhibitor (TKI). It is an orally administered targeted drug which is effective in treating ErbB2-positive metastatic breast cancer. Lapatinib reversibly binds to the intracellular adenosine triphosphate (ATP)-binding

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site of tyrosine kinase domain, thus prevents receptor phosphorylation and activation, hence blocking downstream signalling pathways such as mitogen-activated protein kinase/extracellular-signal regulated kinase (MAPK/Erk) and phosphatidylinositol-3'-kinases (PI3K)/Akt pathways which are involved in cell survival and proliferation. Unfortunately, lapatinib administration is associated with several adverse effects, including diarrhoea [1-2]. The underlying mechanism of lapatinib-induced diarrhoea (LID) remains abstruse. ErbB1 is expressed in the gastrointestinal (GI) mucosa [3] whereby the primary site for drug absorption is the intestine. Therefore, it is hypothesised that ErbB1 TKI administration may interfere with normal GI functioning, resulting in diarrhoea.

ErbB1/EGFR/HER1 is a 170 kDa transmembrane glycoprotein with a single polypeptide chain. ErbB1 is known as a tyrosine kinase member that has a wide signalling pathway including PI3K/Akt, SRC, PLC-γ1/PKC, JNK and JAK/STAT pathways [4]. As such, ErbB1 is able to promote intestinal restitution/wound healing, cell survival, ion transport and other essential elements of mucosal cell physiology [5-6]. However, overexpression of ErbB1 has been correlated to gastric cancer, human hepatocellular carcinoma and oesophageal cancer [7] making ErbB1 inhibition a possible therapeutic approach for treating those tumours. Previous study using ErbB1 TKI, neratinib showed diarrhoea incidence in the *in vivo* model with significant level of interferon-gamma (IFN-δ), an inflammatory cytokine in the ileum [8]. Meanwhile, an *in vitro* study using another ErbB1 TKI, osimertinib also showed higher expression of inflammatory cytokines, where interferon-alpha (IFN-α) and IFN-δ were observed, similarly, the inflammatory cytokines were also expressed in the vivo tumour-bearing model [9]. Besides, lapatinib-treated model also developed diarrhoea with significant tissue changes in jejunum with evidence of intestinal epithelium turnover/maturation caused by interaction with cells expressing ErbB1 and ErbB2 [10]. Thus, these studies basically provide an indispensable relation of ErbB1 and diarrhoea.

Lapatinib, an ErbB1 TKI for metastatic breast cancer treatment, showed diarrhoea as an adverse event in 58% to 78% of patients [2-3]. Investigations on the mechanisms of ErbB1 TKI-induced diarrhoea are currently ongoing, with several conflicting hypotheses presented in the literature, with one of them is believed to be due to ErbB1 inhibition in the small intestine. Even though diarrhoea can be tolerated, it can significantly hamper the quality of life (QOL) of patients with cancer. Severe and potentially life-threatening diarrhoea can implicate in dehydration, bowel discomfort, fatigue and perianal skin breakdown [1]. In addition, lapatinib is usually prescribed for a long term which often causes prolonged diarrhoea. As such, it may result in early mortality, from life-threatening sequelae, or indirectly through changes in cancer treatment which might cause suboptimal therapy [11]. Unequivocally, effective treatment options for LID are critically required, as is the urgent investigation of appropriate mechanisms of LID.

Epidermal growth factor (EGF) is a 53-amino acid polypeptide [12] and can be found in platelets, macrophages and various body fluids such as urine, saliva and amniotic fluid [13-14]. Several studies using EGF proved that EGF functions as gastrointestinal mucosal cryoprotective barrier trophic agent [15] and its proliferative effect is able to being preserved despite the loss of epidermal growth receptor (EGFR) at the enterocytes [16]. Besides, the secretion of EGF protein produced by recombinant *E.coli Nissile 1917* acts as an *in vitro* wound healing agent and subserves the epithelial migration activity in injured human enterocytes monolayers [13]. Hence, EGF would be a potential target for LID.

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In this study, lapatinib is hypothesised to induce diarrhoea through inhibition of ErbB1 in normal GI in which, it will inhibit the tumour cell survival and proliferation in the small intestine. Hence, Caco-2, a human colon adenocarcinoma cell line was chosen as *in vitro* model to study the cytotoxicity of lapatinib since it has similar morphology to normal intestine. In addition, administration of recombinant epidermal growth factor (rEGF) in lapatinib-treated cells, a possible intervention for this study was also investigated to further confirm the involvement of ErbB1 inhibition in LID. Thus, the objectives of this study were to investigate the effect of lapatinib on Caco-2 cell proliferation and to determine the effect of rEGF on Caco-2 cells treated with lapatinib.

2. METHODOLOGY

2.1 Cell culture and reagents

Caco-2 (human colon adenocarcinoma) cell line was purchased from the American Type Culture Collection (ATCC), US. Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM/Ham's F-12), 2 mM L-glutamine, 1 % antibiotic (penicillin-streptomycin) and antimycotic (amphotericin B) mixed solution (Nacalai Tesque, Japan) and supplemented with 10 % foetal bovine serum (Tico Europe, Netherlands). Assays using Caco-2 cell line were carried out between passage 11 to 16. Cells were cultured in 75 cm^2 flasks in a 37 °C incubator with 5 % CO_2. After cell harvesting, cell viability assay was conducted to calculate Caco-2 cell density using Cell Count Reagent SF (Nacalai Tescque, Japan).

About 25 mg of lapatinib powder (Sigma, USA) was dissolved in 100 % dimethyl sulfoxide (DMSO) to a concentration of 17.2096 mM and was further diluted with serum-free DMEM Ham's/F12 medium to a concentration series of 5-100 µM. DMSO was used as vehicle-control and the final concentration of DMSO in cell culture medium was lower than 0.1 %, which is not toxic to the cells. Recombinant epidermal growth factor (rEGF) (Sigma, USA) was reconstituted in 2 ml of 5 % serum in phosphate-buffered saline (PBS). It was then diluted with a serum-free medium to a concentration series of 150-1000 nM.

2.2 Cell viability assay

2.2.1 WST-1 assay

Caco-2 cell proliferation was determined using WST-1 assay. This assay is based on the utilisation of high water-soluble tetrazolium, WST-1 which is able to produce a water-soluble formazan dye upon reduction in presence of electron carriers, which only occurs in viable cells. Then, Caco-2 cells ($1x10^5$ in 0.1 ml complete growth medium) were seeded in each well of a 96-well microtiter flat-bottom plate (Becton Dickinson, USA) and the plate was incubated for 24 hours at 37 °C with 5 % CO_2. After 24 hours incubation, the old media was aspirated and the cells were incubated with a series concentration of lapatinib (5-100 µM) diluted in serum-free medium at four-time points, 24, 48, 72 and 96 hours. An equivalent concentration of DMSO was used as vehicle control, which does not exceed 0.5 %. After the incubation period, 10 µl of cell counting reagent (Cell Count Reagent SF) (Nacalai Tesque, Japan) were added to each well and the plate was incubated again for 1 hour and 30 minutes. The cell viability was measured using a microplate reader (Tecan Infinite M200, Switzerland) at 450 nm using the following formula:

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$$\text{Cell viability (\%)} = \frac{\text{Absorbance}_{450} \text{ value of sample}}{\text{Absorbance}_{450} \text{ value of control}} \times 100\% \quad (1)$$

A graph of percentage of the cell viability versus concentration of lapatinib was then plotted and the IC_{50} values (a dose that inhibited 50 % cell growth) were determined from the graph. The median IC_{50} across 48 to 96 hours were calculated and used for the next experiment.

2.2.2 Intervention with recombinant epidermal growth factor (rEGF)

rEGF was used in this experiment to further confirm the involvement of ErbB1 inhibition. Caco-2 cells ($1x10^5$ in 0.1 ml complete growth medium) were seeded in each well of a 96-well microtiter flat-bottom plate (Becton Dickinson, USA) and the plate was incubated for 24 hours at 37 °C with 5 % CO_2. After 24 hours of incubation, Caco-2 cells were treated with the median IC_{50} value of lapatinib (28 μM) with rEGF from a series concentration of 150 to 1000 nM. Both equivalent concentrations of DMSO and 5 % FBS in PBS were used as vehicle-control in this experiment. The microplate then was incubated at 37 °C, 5 % CO_2, also at four different time-points; 24, 48, 72 and 96 hours. After the incubation period, 10 μl of cell counting reagent (Cell Count Reagent SF) (Nacalai Tesque, Japan) were added to each well and the plate was then incubated again for 1 hour and 30 minutes. The cell proliferation activity was measured using a microplate reader (Tecan Infinite M200, Switzerland) at 450 nm. A graph of percentage of cell proliferation versus concentration of lapatinib+rEGF was then plotted.

2.3 Statistical analysis

All the experiments were repeated independently with a minimum of three times (n=3), and data were expressed as mean±standard deviation (SD). IC_{50} values were analysed using GraphPad Prism 8.0.1. Statistical analyses were accepted at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Lapatinib Induced Toxicity to Caco-2 Cells

In this study, the effect of lapatinib on proliferation of Caco-2 cells was investigated. Lapatinib inhibited cell proliferation in a dose-dependent manner (Figure 1). At 24 hours, lapatinib was not able to inhibit 50 % of cell growth. IC_{50} values were obtained starting at 48 hours (28 μM ± 7.39), 72 hours (29 μM ± 1.45) and 96 hours (14 μM ± 0.95). The median IC_{50} from 48 to 96 hours was then calculated, which was 28 μM ± 2.51. In this study, median was used since the data distribution was skewed. This concentration was then used for combination treatment with rEGF. As for the vehicle-control group, DMSO did not affect the cell inhibition at all time-points (results were not shown). Hence, indicating lapatinib causing direct toxicity to Caco-2 cells, thus supporting the statement that lapatinib inhibits the tumour cell survival and proliferation. Previous cytotoxicity studies of lapatinib on Caco-2 cells revealed various IC_{50} values ranging from 8 μM to 10 μM at 24 and 48 hours respectively [17-18], showing different degrees of lapatinib sensitivity. However, the plausible reason for the result obtained in this study somehow may be affected by the biological characteristics, the establishment of the cell line and the heterogeneity of tumour cells themselves. It is believed that Caco-2 cell line in this experiment might experience changes in the course of cultivation in laboratory conditions, therefore explaining a wide concentration of IC_{50} value obtained in this study compared to

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previous studies. Besides, different incubation time-points and seeding densities might as well explain the variation obtained [19].

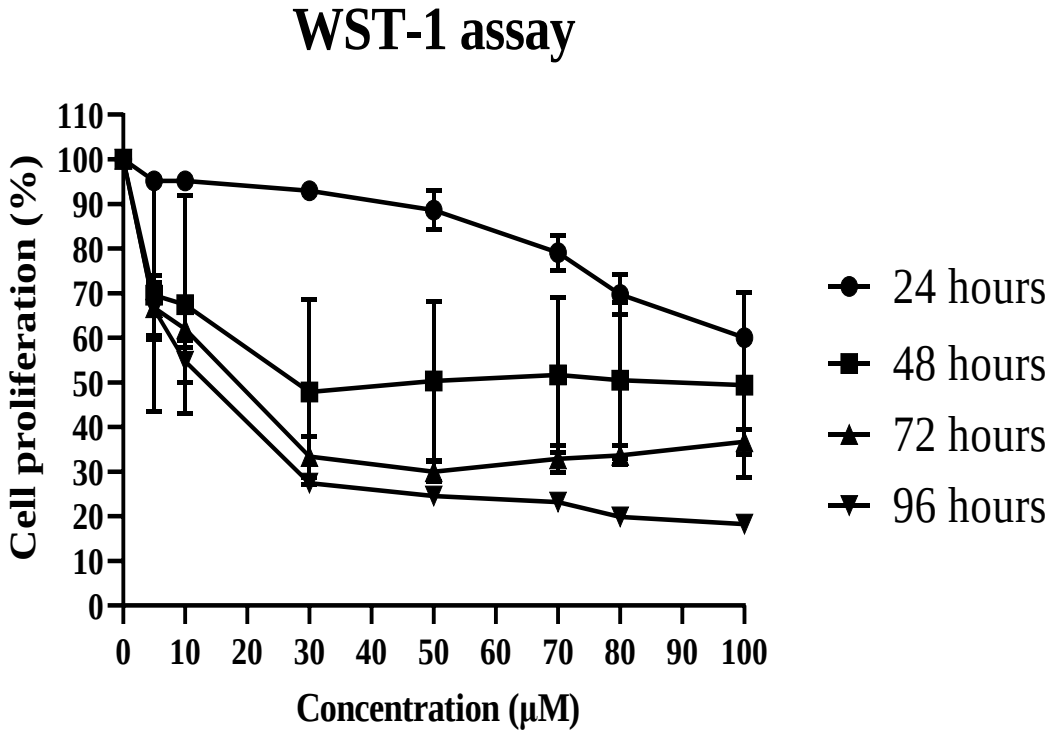

Figure 1: Lapatinib treatment on Caco-2 cells at four time-points; 24, 48, 72 and 96 hours. Lapatinib inhibits 50 % of Caco-2 cell growth at 48 hours (28 µM ± 7.39), 72 hours (29 µM ± 1.45) and 96 hours (14 µM ± 0.95). No inhibition was observed at 24 hours. Results were presented as mean ± SD (*n* = 3).

3.2 Recombinant Epidermal Growth Factor Treatment Promote Cell Proliferation in Caco-2 Cells Treated with Lapatinib

Caco-2 cells were treated with lapatinib, rEGF, lapatinib + rEGF and DMSO + 5 % FBS in PBS (vehicle-control) (Figure 2). Intervention with rEGF at all time-points was found to promote cell proliferation in lapatinib-treated cells. From Figure 2, 150 nM rEGF was able to increase the cell proliferation about 70.8 % ± 5.30 at 24 hours and 56.3 % ± 1.75 at 48 hours. However, at 72 hours, 1000 nM was seem to be able to increase the Caco-2 cell proliferation about 55.95 % ± 7.10 meanwhile at 96 hours, the Caco-2-treated lapatinib was only able to grow about 36.7% ± 5.05 at 500 nM. Median calculated for rEGF across all time-points was 325 nM ± 5.18. However, the fluctuation trend exhibited in this combined effect of ErbB1-TKI and rEGF on Caco-2 cells were unable to be explained. Somehow, it is hypothesised that lapatinib potency is increased over a period of time, especially at 72 hours, and then became less potent at 96 hours due to lower cell proliferation in the lapatinib-treated group. Overall rEGF was observed to be able to induce proliferation of lapatinib-treated cells, hence proving the association of ErbB1 in this experiment. This is supported by a previous study showing rEGF, as a target protein for ErbB1 which proved as a successful proliferative agent for loss of ErbB1 in the enterocytes [13].

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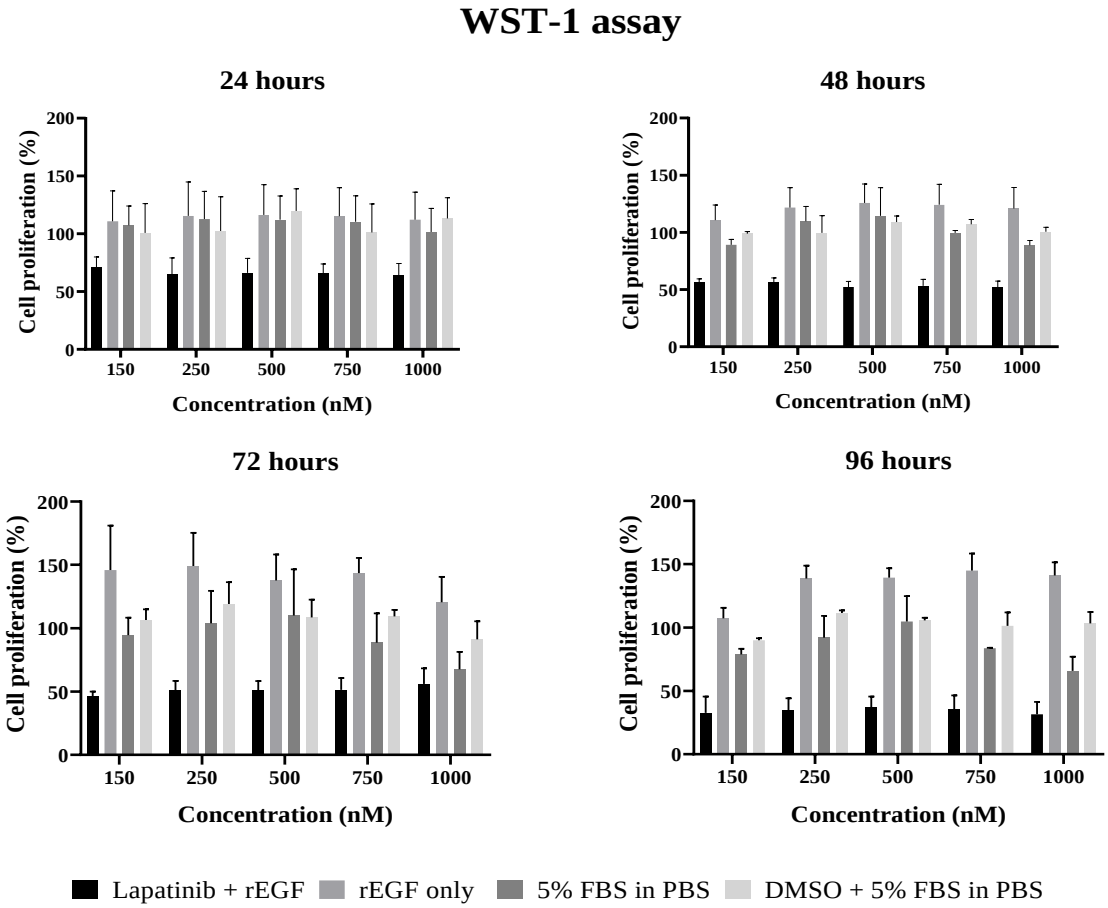

Figure 2: Optimisation of rEGF concentration that could counteract lapatinib action on Caco-2 cells was performed using WST-1 assay. Caco-2 cells were treated with lapatinib, rEGF, lapatinib + rEGF and DMSO + 5 % FBS in PBS (vehicle control) at four time-points, 24, 48, 72 and 96 hours. 150 nM rEGF was able to increase the cell proliferation about 70.8 % ± 5.30 at 24 hours and 56.3 % ± 1.75 at 48 hours. 1000 nM was able to increase the Caco-2 cell proliferation about 55.95 % ± 7.10 at 72 hours while at 96 hours, Caco-2-treated lapatinib was able to grow about 36.7 % ± 5.05 at 500 nM. All results were presented as mean ± SD. r*EGF: recombinant epidermal growth factor; PBS: phosphate buffered saline; FBS: foetal bovine serum; DMSO: dimethyl sulphoxide*

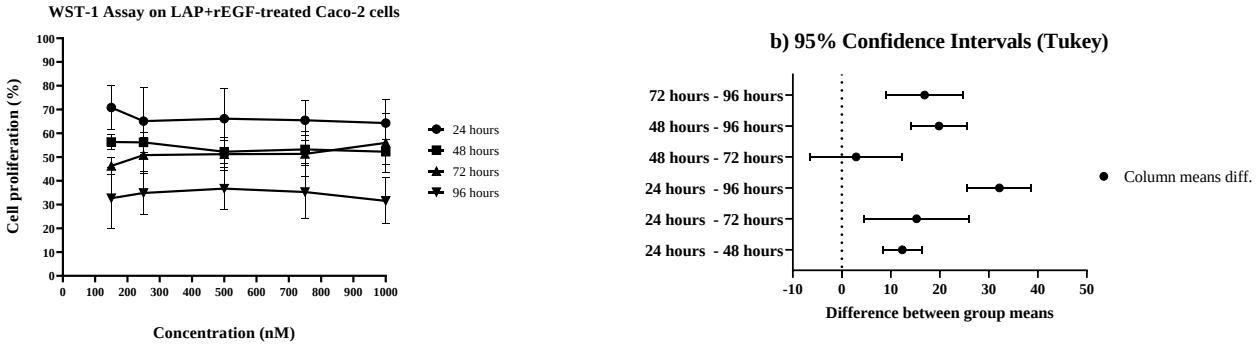

Figure 3: All-time incubation of rEGF in lapatinib-treated Caco-2 cells. The median value for rEGF proliferation at all times was 325 nM ± 5.18. rEGF was able to increase by about 35.5 % of Caco-2 cell proliferation at 325 nM ± 5.18. All results were presented as mean ± SD. Mean incubation time-points were statistically compared using one-way ANOVA using 95 % confidence interval with Tukey test.

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4. CONCLUSION

In summary, this study showed that lapatinib, a successful drug for the treatment of ErbB2-positive breast cancer, exhibits toxicity on the Caco-2 cell line by reducing its proliferation activity. Meanwhile, intervention with rEGF, a proliferative stimulator was able to slightly increase the proliferation of Caco-2 treated with lapatinib, even at higher doses. As far as the authors are concerned, this is the first study which revealed the combination involvement of ErbB1 and rEGF as possible therapeutic target for lapatinib-induced diarrhoea (LID). Even though this is a preliminary study on the mechanism of LID, the results obtained aid to the advances in understanding diarrhoea processes induced by this agent, as such providing clues to the poorly understood processes of ErbB1 TKI-induced diarrhoea. Thus, it is hypothesised that rEGF is capable of subserving the proliferation of lapatinib-treated Caco-2 cells, indirectly supporting the statement of ErbB1 involvement in LID mechanism.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

[1] H. A. Azim *et al.*, "Pattern of rash, diarrhea, and hepatic toxicities secondary to lapatinib and their association with age and response to neoadjuvant therapy: analysis from the NeoALTTO trial," *J. Clin. Oncol.*, vol. 31, no. 36, pp. 4504–4511, 2013.

[2] E. de Azambuja *et al.*, "Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response," *The Lancet Oncology*, vol. 15, no. 10, pp. 1137–1146, 2014.

[3] K. R. Secombe, Y. Z. A. Van Sebille, B. J. Mayo, J. K. Coller, R. J. Gibson, and J. M. Bowen, "Diarrhea Induced by Small Molecule Tyrosine Kinase Inhibitors Compared With Chemotherapy: Potential Role of the Microbiome," *Integr Cancer Ther*, vol. 19, 2020.

[4] A. Appert-Collin, P. Hubert, G. Crémel, and A. Bennasroune, "Role of ErbB Receptors in Cancer Cell Migration and Invasion," *Front. Pharmacol.*, vol. 6, p. 283, 2015.

[5] M. R. Frey and D. Brent Polk, "ErbB receptors and their growth factor ligands in pediatric intestinal inflammation," *Pediatr Res*, vol. 75, no. 1–2, pp. 127–132, 2014.

[6] P. Wee and Z. Wang, "Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways," *Cancers*, vol. 9, no. 5, p. 52, 2017.

[7] E. Rossi *et al.*, "HER-2 overexpression/amplification in Barrett's oesophagus predicts early transition from dysplasia to adenocarcinoma: a clinico-pathologic study," *J Cell Mol Med*, vol. 13, no. 9b, pp. 3826–3833, 2009.

[8] K. R. Secombe *et al.*, "Targeting neratinib-induced diarrhea with budesonide and colesevelam in a rat model," *Cancer Chemother Pharmacol*, vol. 83, no. 3, pp. 531–543, 2019.

[9] N. J. Gurule *et al.*, "A tyrosine kinase inhibitor-induced interferon response positively associates with clinical response in EGFR-mutant lung cancer," *NPJ Precis Oncol*, vol. 5, no. 1, p. 41, 2021.

p-ISSN 1675-7939; e-ISSN 2289-4934

[10] J. M. Bowen *et al.*, "Determining the mechanisms of lapatinib-induced diarrhoea using a rat model," *Cancer Chemother Pharmacol*, vol. 74, no. 3, pp. 617–627, 2014.
[11] G. Richardson and R. Dobish, "Chemotherapy induced diarrhea," *J Oncol Pharm Pract*, vol. 13, no. 4, pp. 181–198, 2007.
[12] J. Berlanga-Acosta, Y. Mendoza-Mari, A. Garcia-Ojalvo, J. A. Acosta-Buxado, M. Fernandez-Mayola, and G. Guillen Nieto, "Epidermal Growth Factor (EGF) intralesional infiltrations: From the bench to the diabetic ulcers cells," *Integr Mol Med*, vol. 6, no. 1, pp. 1-7, 2019.
[13] H. J. Choi, J. H. Ahn, S.-H. Park, K. H. Do, J. Kim, and Y. Moon, "Enhanced Wound Healing by Recombinant Escherichia coli Nissle 1917 via Human Epidermal Growth Factor Receptor in Human Intestinal Epithelial Cells: Therapeutic Implication Using Recombinant Probiotics," *Infection and Immunity*, vol. 80, no. 3, pp. 1079–1087, 2012.
[14] X. Tang, H. Liu, S. Yang, Z. Li, J. Zhong, and R. Fang, "Epidermal Growth Factor and Intestinal Barrier Function," *Mediators Inflamm*, vol. 2016, pp. 1-10, 2016.
[15] P. Arda-Pirincci and S. Bolkent, "The role of epidermal growth factor in prevention of oxidative injury and apoptosis induced by intestinal ischemia/reperfusion in rats," *Acta Histochemica*, vol. 116, no. 1, pp. 167–175, 2014.
[16] K. J. Rowland, P. M. Choi, and B. W. Warner, "The Role of Growth Factors in Intestinal Regeneration and Repair in Necrotizing Enterocolitis," *Semin Pediatr Surg*, vol. 22, no. 2, pp. 101–111, 2013.
[17] E. Giannopoulou, A. Antonacopoulou, K. Floratou, A. G. Papavassiliou, and H. P. Kalofonos, "Dual targeting of EGFR and HER-2 in colon cancer cell lines," *Cancer Chemother Pharmacol*, vol. 63, no. 6, pp. 973–981, 2009.
[18] Z. Xiang *et al.*, "LncRNAs GIHCG and SPINT1-AS1 Are Crucial Factors for Pan-Cancer Cells Sensitivity to Lapatinib," *Front Genet*, vol. 10, p. 25, 2019.
[19] Y. He *et al.*, "The changing 50% inhibitory concentration (IC50) of cisplatin: a pilot study on the artifacts of the MTT assay and the precise measurement of density-dependent chemoresistance in ovarian cancer," *Oncotarget*, vol. 7, no. 43, pp. 70803–70821, 2016.

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