Conjugation of Cetuximab – Puromycin and Its Target-Specific Effect on Triple Negative Breast Cancer Cell Lines

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Abstract

Cancer is life-threatening disease and being global health problems. Chemotherapy is one of the most used therapy for cancer since many years ago. Chemotherapy is also toxic for normal cell, not specific to the target cells. Consequently, chemotherapy has various side effects. Monoclonal antibody (MAb) has been developed for specific therapy which only has killing effect in cancer cells, but the survival rate of most MAbs around 20%. Therefore, in clinical practice, MAbs administration should combine with chemotherapeutic agents. For effectiveness of therapy and to minimize adverse effects, anticancer agent with selective cytotoxic effect on target cells is needed, the immunotoxin. Objective: This study introduces a novel approach to conjugate monoclonal antibody (Cetuximab) and toxin (Puromycin), in order to selectively inhibit proliferation of triple negative breast cancer (TNBC) and to enhance the efficacy of MAb in target cells killing. Methods: Cetuximab was conjugated with Puromycin using a linker, i.e SATP (Succinimidyl-acetylthiopropionate) and tested on triple negative breast cancer cell lines (MDA-MB-231) which expressed EGFR (epidermal growth factor receptor). Cetuximab is MAb which targets EGFR. MCF-7 was used as control cells since it has low or no EGFR expression. Cell counting were conducted as viability assay at 24 hours, 48 hours, and 72 hours after treatment. Results: The results showed significant reduction of live cells number in Conjugate 20 µg/mL cultured in MDA-MB-231 compared to MCF-7 after 24 hours, 48 hours, and 72 hours incubation. In all time period of incubation, significant reduction of MDA-MB-231 live cells number was also observed in Conjugate 20 µg/mL compared to Cetuximab 20 µg/mL. Conclusion: Synthesized conjugate showed its target-specific effect in TNBC and improved the efficacy of Cetuximab on TNBC. In the future, this conjugate can be a potential anticancer therapy in treating triple-negative breast cancer.

Keywords: Triple negative breast cancer- cetuximab- puromycin- MDA-MB-231- immunotoxin

Introduction

Cancer is a life-threatening disease which become a global health problem. Breast cancer is the most common cancer type in women (WHO, 2020). Breast cancer has various types, one of them is triple-negative breast cancer (TNBC). TNBC is basal subtype which does not express estrogen and progesterone receptors, and lacks HER-2 expression, but highly express EGFR (epidermal growth factor receptor) on the cell surface (Mueller et al., 2010). TNBC is very aggressive and could divide and spread more quickly. The therapeutic option for TNBC is limited, furthermore TNBC has a poor prognosis (Podo et al., 2010).

Currently, there are several therapeutic options for TNBC, including surgery, radiation, chemotherapy, and monoclonal antibodies. Surgery, for tumor removal, has been considered unsuccessful due to recurrence of the cancer (Schirrmacher, 2019). On the other hand, radiotherapy significantly decrease patient’s quality of life, such as nausea and vomiting, hair loss. Radiotherapy is not specific and has detrimental effects on normal cells (Aruna, 2006). Chemotherapy is toxic to cells (Malhotra et al., 2003) which inhibits DNA replication and mitosis (Cattley et al., 2004). Similar to radiotherapy, chemotherapy kills the target cells nonspecifically. thus causing extensive damage to normal cells, especially in cells with high proliferative rates such as bone marrow (Agarwal, 2016) and gastrointestinal tract (Ramirez et al., 2009). It leads to morbidity or even mortality. In addition, chemotherapy causes resistance (Luqmani, 2005).

Monoclonal antibodies (MAbs) indicated for TNBC have been approved by several regulatory authorities (Park et al., 2018). Although MAbs bind to specific receptor on the target cells (Aruna, 2006), but, in order to kill cancer, immune cells should be involved. In other words, use of MAbs is less curative (Scott et al., 2012; Shah et al., 2018). Moreover, the survival rate of most MAbs around 20%.

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For that reason, MAbs administration should combine with chemotherapeutic agents in clinical practice (Park et al., 2018). For effectiveness of therapy and to minimize the adverse effects, anticancer agent with selective cytotoxic effect on target cells is needed, the immunotoxin.

Immunotoxin is the conjugation between antibody and toxin (Aruna, 2006). Some immunotoxin have been developed, for instance amanitin-Mab and diphteria toxin-Mab conjugates. As both are very toxic compound which can work selectively, we wonder if another toxic substance can be coupled to a specific Mab and use as targeted immunotoxin. We considered to use another toxin, Puromycin to be conjugated with Cetuximab through a linker, SATP (N-Succinimidy l S-Acetylthiopropionate). Cetuximab, specific for TNBC is a chimeric monoclonal antibody which has a humanized Fc moiety, so that better immunogenicity profile compared to murine antibodies (Li et al., 2005).

Puromycin, a compound from Streptomycyes alboniger (Nathans, 1964), was chosen as a cytotoxic component of immunotoxin in this study as it causes premature termination during translation at the ribosome (Nathans, 1964). Puromycin is known to induce apoptosis in breast cancer cells through insulin-like growth factor-1 (IGF-1) and has antitumor activity on MDA-MB-231 through suppression of 45S pre-ribosomal RNA and upstream binding factor (UBF) (Jung et al., 2013; Söderlund et al., 2004). Puromycin is also known to exhibit p53-dependent apoptotic effects on human colorectal carcinoma cells (Jung et al., 2019).

The MDA-MB-231 is a triple-negative breast cancer cell lines (LaChance MP et al., 1984). From immunohistochemical analysis, MDA-MB-231 showed high expression of EGFR on the cell surface (Greg, 2008). Since this type of cancer has the worst prognosis amongst all types of breast cancer with the limited treatment choices and the current treatment especially chemotherapy/radiotherapy cause therapy resistance, recurrence, side effects, and the targeted therapy like MAb needs combination of chemotherapy/radiotherapy (Podo et al., 2010), through this study, the immunotoxin, conjugate Cetuximab – Puromycin, is expected to selectively inhibit the proliferation of triple-negative breast cancer cells (MDA-MB-231) and enhance the efficacy of Cetuximab in killing target cells.

Materials and Methods

Materials

Cetuximab 5 mg/mL (Erbitux, Merck); Puromycin (Thermo); N-Succinimidyl S-Acetylthiopropionate/ SATP (Thermo); Dialysis tubing cellulose membrane (Sigma Aldrich); Sephadex G-100 SF (Pharmacia Fine Chemicals); Hydroxylamine hydrochloride (Sigma Aldrich); MDA-MB-231 (Faculty of Medicine, Universitas Indonesia); Cell Counter Plate (LUNA).

Preparation of Cetuximab

In order to remove small molecules, Cetuximab aqueous solution (5 mg/mL) was dialyzed in 50 mM phosphate buffer (pH 7.5) which contains 1 mM EDTA. It was kept for 12 hours at 4°C (Hajighasemlou et al., 2015). MR Cetuximab = 145,781.6 Da.

SATP is soluble in organic solvent, i.e. DMSO. Immediately, before reaction, 3.7 mg of SATP was dissolved in 0.23 mL of DMSO at concentration of 65 mM (16 mg/mL). In the ratio of 100: 1 (protein solution 10 mg/mL: SATP solution), 5 µL SATP was added to each mL of Cetuximab 5 mg/mL. They are mixed and incubated at room temperature for 60 minutes. Modified Cetuximab was separated from unreacted SATP and reaction-by-products by dialysis against 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA (Greg, 2008).

Acetylated -SH group was deprotected by adding 50 µL of 50 mM Hydroxylamine hydrochloride dissolved in 50 mM phosphate buffer (pH 7.5) and contains 1 mM EDTA to each mL of SATP-modified protein solution. The mixture was incubated for 2 hours at room temperature. Sulphhydryl-modified Cetuximab was purified by dialysis against 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA.

Preparation of Puromycin

Puromycin was dissolved at concentration 5 mg/mL in 50 mM phosphate buffer (pH 7.5) which contains 1 mM EDTA. To remove small molecules, Puromycin was dialyzed against 50 mM phosphate buffer (pH 7.5) contains 1 mM EDTA. It was kept for 12 hours at 4°C (Hajighasemlou et al., 2015). Puromycin was prepared according to the preparation of Cetuximab.

Moreover, in order to know maximum wavelength of Puromycin, Puromycin was dissolved at concentration 0.5 mg/mL and measured in Spectrophotometer UV-Vis in the wavelength of 190 – 400 nm. Figure 3 showed that the maximum wavelength of Puromycin was 270 nm.

Preparation of Immunotoxin

500 µL activated Puromycin was added to the 250 µL of activated Cetuximab (molar ratio 2:1) in a microtube and the mixture was wrapped with aluminum foil and kept for 18 hours at room temperature (Hajighasemlou et al., 2015).

Conjugate was purified by gel filtration on a Sephadex-100 SF column using the same buffer, phosphate – EDTA. Eluates from the peak were collected to be dialyzed against the same phosphate – EDTA buffer for 12 hours at 4°C. External buffer was checked for absorbance at a wavelength of 270 nm (max. wavelength for Puromycin). The concentration of conjugate from dialysate was measured at 280 nm using Albumin as standard.

Conjugate Confirmation

In order to confirm the existences of Cetuximab and Puromycin in synthesized conjugate, absorbance of conjugate was measured at 270 nm and 280 nm compared to control, Cetuximab and Puromycin, in the same concentration (20 µg/ml). Wavelength of 270 nm was maximum wavelength for Puromycin and 280 nm was maximum wavelength for Cetuximab as a protein.
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Cell Lines Culture
MDA-MB-231 and MCF-7 were cultured in the flask at temperature 37°C, 5% CO₂, DMEM high glucose, 10% FBS (fetal bovine serum), 1% Penicillin-Streptomycin, and 1% Amphoterin were used as complete medium to feed cell lines and maintain cell growth. The culture medium was replaced every 72 hours. MDA-MB-231 was used as test cells, meanwhile MCF-7 as control cells.

Cell Proliferation Assay
MDA-MB-231 and MCF-7 were dissociated from cell flasks by Trypsin digestion and seeded into 24-well plates, 50,000 cells/well. Cells were harvested by Trypsin and counted each day using trypan blue excursion method. Cell growth curves were drawn from live cell numbers for seven days.

Statistical Analysis
Live cells number from viability assay was counted and analyzed nonparametrically by Mann-Whitney U test by using SPSS 25. Statistical significance was assumed at p < 0.05.

Results
Conjugate was purified by size exclusion chromatography (SEC) using Sephadex G-100 SF to separate the conjugate from the excess of Puromycin and reaction-by-products, since in the conjugation reaction Puromycin molarity exceeded the Cetuximab (2:1). Purification was done three times (see Figure 4) and gave consistent 3 peaks and patterns. Eluates were collected and furthermore concentrated by dialysis until external buffer of dialysis had no absorbance at 270 nm (max. wavelength for Puromycin). The concentration of dialyzed conjugate was 25.84 mg/mL, measured as protein at 280 nm.

The synthesized conjugate was confirmed for the existences of Cetuximab and Puromycin on its structure by Spectrophotometer UV-Vis. The results were shown in Table 1. At the same concentration (20 µg/ml), absorbance of conjugate at 280 nm showed similarity in compared to Cetuximab. Furthermore, the result at wavelength of 270 nm supported the presence of 0.25% Trypsin and then incubated in 37°C, 5% CO₂ for 10 hours. Hereafter, complete medium was added to the cells in order to stop Trypsin activity. Cell suspension was transferred to the sterile tube and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded, but pellet was being resuspended in 1 mL medium. Ten µL were mixed with 10 µL Trypan blue to stain the cells. Cell counting was conducted by LUNA cell counter.

Cytotoxicity Assay/CC50
CC50 defined as as the concentration of tested substance (in this case Cetuximab – Puromycin conjugate) which kill 50% cell population. Cells were seeded into 6-well plates, 500,000 cells/well. One day (24 hours) before treatment, MDA-MB-231 cells are being fasted from FBS. Cells are divided into five different doses of conjugate (5 µg/mL; 10 µg/mL; 20 µg/mL; 40 µg/mL; 80 µg/mL) and one untreated group. They were incubated for 48 hours. The viable cell number was determined by cell counting after trypan blue excursion method. The CC50 against this cell was determined from dose-response curve.

Viability Assay
For treatment purpose, cells were seeded into 24-well plates, 50,000 cells/well. One day (24 hours) before treatment, cell lines are being fasted from FBS. MDA-MB-231 and MCF-7 cell lines were divided into eight groups:
- Group A: MDA-MB-231 + Conjugate of Cetuximab and Puromycin 10 µg/mL
- Group B: MDA-MB-231 + Conjugate of Cetuximab and Puromycin 20 µg/mL
- Group C: MDA-MB-231 + Conjugate of Cetuximab and Puromycin 40 µg/mL
- Group D: MDA-MB-231 + Cetuximab 20 µg/mL
- Group E: MDA-MB-231 + Puromycin 20 µg/mL
- Group F: MDA-MB-231 + Placebo
- Group G: MCF-7 + Conjugate of Cetuximab and Puromycin 20 µg/mL
- Group H: MCF-7 + Placebo

The viability assay was done on 24 hours, 48 hours, and 72 hours after treatment. Cells were harvested by
Puromycin structure in synthesized conjugate.

MDA-MB-231 is epithelial, human breast cancer cell lines which has spheroid structure (see Figure 5). It is a highly aggressive, invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell line as it does not have of estrogen receptor (ER), progesterone receptor (PR), and lacks of HER2 (human epidermal growth factor receptor 2), but highly express EGFR (epidermal growth factor receptor) on the cell surface. MCF-7 has an epithelial-like morphology and monolayers for dome structure (see Figure 4). MCF-7 has no or lacks of EGF receptor. Figure 4 was taken 5 days after culture in the flask.

To compare the rate of proliferation between MDA-MB-231 and MCF-7, cell numbers were counted each day for seven days, and live cells number were used for cell growth curves (see Figure 6). Descriptively, MCF-7 showed lower growth rate than MDA-MB-231.

The results of cytotoxicity assay/CC50 were shown in Figure 7. Percentage of response was described as percentage of the cell death in groups with different doses compared to untreated group. As formula, it can be counted as [(cell numbers on untreated group – cell numbers on treated group) / cell numbers on untreated group] x 100.

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**Table 1. Absorbance of Conjugate Compared to Cetuximab and Puromycin at Wavelength of 280 nm and 270 nm.**

|                | absorbance at 280 nm | absorbance at 270 nm |
|----------------|----------------------|----------------------|
| conjugate      | 0.267                | 0.63                 |
| cetuximab      | 0.211                | 0.355                |
| puromycin      |                      | 0.246                |

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Figure 4. Purification Chromatogram of Conjugate which Performed by SEC Sephadex G-100 SF (Mobile Phase: 50 mM phosphate buffer pH 7.5 which contains 1 mM EDTA)
numbers on test group)/cell numbers on untreated group) x 100%. From the linear regression calculation, an equation for a correspondent straight line was formulated as \( y = 0.9857x + 13.115, \ R^2 = 0.9955 \). Hence, to get 50% cytotoxicity concentration of the conjugate, \( y = 50 \) was added to the formula. In consequence, CC50 of the conjugate was 37.42 µg/mL.

Viability was measured as live cell number after

![Figure 5. Cell Colonies of MDA-MB-231 (left) and MCF-7 (right) in 40X Microscopes Magnification](image)

![Figure 6. Cell Proliferation Growth Curve of MDA-MB-231 and MCF-7](image)

![Figure 7. Dose-Response Curve to Assess CC50 of Conjugate on MDA-MB-231](image)
24-hour, 48-hour, and 72-hour of treatment. Results of 24-hour incubation was showed in Figure 8, Conjugate 20 µg/mL showed significant reduction of live cells number compared to Placebo (p < 0.05, p = 0.025), as well as to the Cetuximab 20 µg/mL (p = 0.05, p = 0.034) and to the Conjugate 20 µg/mL cultured in MCF-7 (p < 0.05, p = 0.025). Results of 48-hour incubation was showed in Figure 9, Conjugate 20 µg/mL showed significant reduction of live cells number compared to Placebo (p < 0.05, p = 0.046), as well as to the Cetuximab 20 µg/mL (p = 0.05, p = 0.034) and to the Conjugate 20 µg/mL cultured in MCF-7 (p < 0.05, p = 0.025). (p < 0.05 defined as statistically significant)
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**Discussion**

Puromycin was conjugated to Cetuximab chemically through a linker, SATP (N-Succinimidyl S-Acetylthiopropionate), by utilizing free amine group (-NH2) in those compounds. Cetuximab has various amino acid in light and heavy chains, including amino acid which have free amine groups, such as lysine (K), arginine (R), asparagine (N), and glutamine (Q) (Ayoub et al., 2013). Puromycin also has free amine group (see Figure 2) on its structure.

SATP retained all the advantages of a protected sulphydryl, including stability of the modified protein.
and selective release of the protecting group with hydroxylamine to free the sulphydryl as needed (Greg, 2008). The deacetylated molecule was used immediately to prevent loss of sulphydryl group through disulfide formation by oxygen. This linker added -SH group on N-terminal at the completion of peptide synthesis. SATP and modified compounds were reacted by making a peptide bond (-NH-CO-) (see Figure 11). Additionally, SATP used in the acylation reaction was calculated precisely because higher levels of acylation correspond to greater risk of protein inactivation although more complete acylation of all primary amino groups will occur when larger molar excesses of SATP are used (Greg, 2008).

The activation of sulphydryl group on Puromycin is according to the Cetuximab. Molar ratio of 2 : 1 (thiolated Puromycin : thiolated Cetuximab) was used to make sure that Cetuximab was conjugated to Puromycin. Immunotoxin made by chemical conjugation mimics the natural linkage between two subunit toxin (A and B subunit) like in diptheria toxin, which connected by disulfide bonds (Ghetie. et al., 2001; Greg, 2008). Therefore, Puromycin was conjugated to Cetuximab by forming disulfide bonds between thiolated Cetuximab and thiolated Puromycin which activated by SATP and hydroxylamine. Amine group in Puromycin were not utilized directly (without activation by SATP and hydroxylamine) to react with thiolated Cetuximab due to prevention the formation of thioether bonds. The previous study found that enzyme in human cells could not hydrolyze thioeter bonds (Ghetie. et al., 2001).

Size exclusion chromatography in Sephadex G-100, followed by extensive dialysis assured purification of conjugate, because the conjugated puromycin eluted in the large molecule fraction and the free puromycin were removed from the eluate by dialysis. All of these were confirmed spectrophotometrically.

MDA-MB-231 is TNBC cell lines which does not express estrogen and progesterone receptors, and lacks HER-2 expression, but highly express or tend to overexpress EGFR (epidermal growth factor receptor) on the cell surface (Bianchini et al., 2016; Moon et al., 2009; Mueller et al., 2010). In order to prove the target-specific effect of synthesized conjugate on TNBC cell lines, MCF-7 were used as control cell since MCF-7 express low EGFR (Moon et al., 2009). The result in Figure 5 showed that proliferation rate of MDA-MB-231 was higher than MCF-7. This is due to the growth and proliferation characteristic of MDA-MB-231.

Cytotoxicity assay/CC50 assessed the conjugate concentration that reduced MDA-MB-231 cell viability by 50% when compared to untreated controls, which were calculated by regression equation. CC50 of the synthesized conjugate was 37.42 µg/mL. This result supported doses selection for viability assay.

Viability was measured as live cell number after 24-hour, 48-hour, and 72-hour of treatment. Conjugate 20 µg/mL showed significant reduction of live cells number compared to Placebo after 24 hours, 48 hours, and 72 hours incubation. In all time period of incubation, significant reduction of live cells number was also
observed in Conjugate 20 µg/mL compared to Cetuximab 20 µg/mL. It showed that synthesized conjugate had better efficacy than Cetuximab at the same concentration. The significant reduction of live cells number in Conjugate 20 µg/mL cultured in MDA-MB-231 compared to MCF-7 showed target-specific effect of synthesized conjugate on TNBC cell lines, after 24 hours, 48 hours, and 72 hours incubation.

Puromycin 20 µg/mL cultured in MDA-MB-231 had no live cells number at all period time of incubation. Puromycin is not selective in cell killing (Nathans, 1964). The previous study showed that Puromycin which labeled with fluorescence can easily cross cell membranes in a nondestructive fashion in mammalian cells (Starck et al., 2004). Meanwhile, synthesized conjugate needs specific EGF receptor on the cell surface to enter cells.

Cetuximab-contained in conjugate directs Puromycin selectively to triple-negative breast cancer through EGF receptor binding. Cetuximab binds the ectodomain of the EGF receptor and consequently block the specific ligand binding and intracellular signaling pathways (Liao H-J et al., 2010). Therefore, PI3K/Akt/mTOR pathway are inhibited (Wang et al., 2021). This binding also initiates receptor endocytosis (Liao et al., 2010). The initiation of receptor endocytosis make Puromycin carried by Cetuximab enters the cell. Synthesized conjugate was held together by disulfide bond. As illustration according to the literature (Kim et al., 2020), in the endocytic compartment, conjugate is cleaved by protease and the disulfide bond is reduced, thus Puromycin is trafficked by late endosome to the trans-golgi network (TGN), and there binds to the KDEL receptor, which traffics it to the endoplasmic reticulum (ER). From there, Puromycin is translocated into cytosol and move to ribosome to cause premature termination of translation process (see illustration in Figure 12).

Puromycin has similar chemical structure of aminoacyl-tRNA. Puromycin will occupy the ribosome site A and bind to the polypeptide chain synthesized by the enzyme peptidyl transferase thereby inhibiting the further entry of aminoacyl-tRNA. The weak binding of puromycin to the site A ribosomes causes the polypeptide chain to break and protein synthesis stops prematurely. Ribosome site A is the site where the codon and anticodon (of aminoacyl-tRNA) interact. This interruption by Puromycin will prevent the codon from pairing with an anticodon (Nathans, 1964). Puromycin was known to induce apoptosis in breast cancer cells by insulin-like growth factor 1 (IGF-1) and exert antitumor activity in MDA-MB-231 via suppression of 45S pre-ribosomal RNA and upstream binding factor (UBF) (Jung et al., 2013; Söderlund et al., 2004). Puromycin was also shown to have p53-dependent apoptotic effect in human colorectal carcinoma cell through HCT116 cell lines (Jung et al., 2019).

This study has the following limitations. Cetuximab was not used as a control during purification by SEC, so that chromatogram peak of the conjugate Cetuximab – Puromycin was not compared to the Cetuximab. In addition, the absorbances of three different fractionations were not measured separately. It would be better if CC50 of Cetuximab could be assessed in compared to the CC50 of the conjugate Cetuximab – Puromycin.

In the future, conjugate Cetuximab – Puromycin can be a potential anticancer therapy in treating triple-negative breast cancer. Apoptotic signaling inside the cells needs further investigation. Other than that, synthesized conjugate may be further characterized to have better evaluation its anticancer potential against triple negative breast cancer.

**Author Contribution Statement**

Diah Puspitasari contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript. Septelia I. Wanandi and Mohamad Sadikin devised the project, the main conceptual ideas, and the design of the research, as well as analyzed and discussed the results.

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**Approval from Scientific Body**

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**Ethical Issue**

Not applicable.

**Statement Conflict of Interest**

The authors declare that there is no conflict of interest.

**References**

Agarwal MB (2016). Is cancer chemotherapy dying?. Asian J Transfus Sci, 10, S1-7.

Aruna G (2006). Immunotoxins: a review of their use in cancer treatment. J Siem Cells Regen Med, 1, 31-6.

Ayoub D, Jabs W, Resemann A, Evers W, et al (2013). Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. MAbs, 5, 699-710.

Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L (2016). Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol, 13, 674-90.

Cattley RC, Radinsky RR (2004). Cancer therapeutics: understanding the mechanism of action. Toxicol Pathol, 32, 116-21.

Ghetie, V, Vitetta ES (2001). Chemical construction of immunotoxins. Mol Biotechnol, 18, 251-68.

Greg T (2008). Herrmannso. Bioconjugate Techniques: Immunotoxin Conjugation Techniques. 2nd Edito. Rockford, Illinois, USA: Pierce Biotechnology, Thermo Fisher Scientific, PP 824-57.

Hajighasemlou S, Alebouyeh M, Rastegar H, et al (2015).
Preparation of immunotoxin herceptin-botulinum and killing effects on two breast cancer cell lines. Asian Pac J Cancer Prev, 16, 5977-81.

Jung JH, Lee H, Kim JH, et al (2019). p53-Dependent Apoptotic Effect of Puromycin via Binding of Ribosomal Protein L5 and L11 to MDM2 and its Combination Effect with RITA or Doxorubicin. Cancers (Basel), 11, 1-13.

Jung JH, Sohn EJ, Shin EA, et al (2013). Melatonin suppresses the expression of 45S preribosomal RNA and upstream Binding Factor and Enhances the Antitumor Activity of puromycin in MDA-MB-231 breast cancer cells. Evid Based Complement Alternat Med, 2013, 879746.

Kim JS, Jun SY, YS K (2020). Critical issues in the development of immunotoxins for anticancer therapy. J Pharm Sci, 109, 104-15.

LaChance MP, Schultz GS, SL F (1984). Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res, 44, 3442-7.

Li S, Schmitz KR, Jeffrey PD, et al (2005). Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell, 7, 301-11.

Liao H-J, Carpenter G (2010). Cetuximab/C225 -Induced Intracellular Trafficking of EGF Receptor. Cancer Res, 69, 6179-83.

Luqmani YA (2005). Mechanisms of drug resistance in cancer chemotherapy. Med Princ Pract, 14, 35-48.

Malhotra V, Perry MC (2003). Classical chemotherapy: mechanisms, toxicities and the therapeutic window. Cancer Biol Ther, 2, 2-4.

Moon DO, Kim MO, Heo MS, et al (2009). Gefitinib induces apoptosis and decreases telomerase activity in MDA-MB-231 human breast cancer cells. Arch Pharm Res, 32, 1351-60.

Mueller KL, Yang ZQ, Haddad R, et al (2010). EGFR/Met association regulates EGFR TKI resistance in breast cancer. J Mol Signal, 5, 8.

Nathans D (1964). Inhibition of protein synthesis by Puromycin. Fed Proc, 23, 984-9.

Park JH, Ahn JH, Kim SB (2018). How shall we treat early triple-negative breast cancer (TNBC): from the current standard to upcoming immuno-molecular strategies. ESMO Open, 3, e000357.

Podo F, Buydens LM, Degani H, et al (2010). Triple-negative breast cancer: present challenges and new perspectives. Mol Oncol, 4, 209-29.

Ramirez LY, Huestis SE, Yap TY, et al (2009). Potential chemotherapy side effects: what do oncologists tell parents?. Pediatr Blood Cancer, 52, 497-502.

Schirrmacher V (2019). From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review). Int J Oncol, 54, 407-19.

Scott AM, Allison JP, Wolchok JD (2012). Monoclonal antibodies in cancer therapy. Cancer Immun, 12, 14.

Shah NJ, Kelly WJ, Liu SV, Choquette K, Spira A (2018). Product review on the Anti-PD-L1 antibody atezolizumab. Hum Vaccin Immunother, 14, 269-76.

Söderlund G, Haarhaus M, Chisalita S, Arnqvist HJ (2004). Inhibition of puromycin-induced apoptosis in breast cancer cells by IGF-I occurs simultaneously with increased protein synthesis. Neoplasma, 51, 1-11.

Starck SR, Green HM, Alberola-Ila J, Roberts RW (2004). A general approach to detect protein expression in vivo using fluorescent puromycin conjugates. Chem Biol, 11, 999-1008.

Wang Z, Goto Y, Allevato MM, et al (2021). Disruption of the HER3-P13K-mTOR oncogenic signaling axis and PD-1 blockade as a multimodal precision immunotherapy in head and neck cancer. Nat Commun, 12, 2383.

WHO (2020). Cancer Breast cancer. Breast Cancer [Internet]. Has been downloaded on 14 Februari 2020, 23:27. Available from: https://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/.

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