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REVIEW

A review of the relationship between Doxorubicin and Doxorubicinol, CBR1 polymorphism, and cardiotoxicity

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Abstract

Doxorubicin is a chemotherapy drug given to breast cancer patients. However, their administration is limited by their cardiotoxicity. The CBR1 enzyme in the liver catalyses doxorubicin to doxorubicinol. Doxorubicinol also contributes to the cardiotoxicity of doxorubicin. Doxorubicin and doxorubicinol levels in the body are affected by the polymorphism of the CBR1 enzyme. **Objective:** To review the effect of CBR1 polymorphisms on the levels of doxorubicin and doxorubicinol after administration of doxorubicin. **Methods:** Relevant studies from selected databases were examined; Three main studies with 20 support studies were reviewed. **Results:** The recommended methods were the analysis of doxorubicin and doxorubicinol levels using the Dried Blood Spot biosampling technique, which uses the ultra-high-performance liquid chromatography-tandem mass spectrometry (LCMS/MS), and the evaluation of the genetic profile of CBR1 using Polymerase Chain Reaction. **Conclusion:** Four CBR1 genetic polymorphisms have been shown to reduce doxorubicinol levels in the body, which is associated with decreased CBR1 activity and expression. Thus, the conversion of doxorubicin to doxorubicinol is reduced. Therefore, individuals who experience CBR1 polymorphisms have a lower risk of cardiotoxicity after the administration of doxorubicin.

Introduction

Breast cancer is a type of cancer that is common among women around the world. Around 10% of cases are hereditary and associated with a family history. Breast cancer can also be caused by a genetic predisposition in the form of mutations in the BRCA1 and BRCA2 genes, as well as patient lifestyle interventions (WHO, 2018; Asia WHOS, 2019; Harbeck *et al*., 2019).

One of the cytotoxic drugs for treatment in breast cancer patients is doxorubicin. Doxorubicin is an anti-cancer drug in the anthracycline group. Its action mechanism forms a complex with DNA and topoisomerase II enzymes that affect DNA replication (Zhao *et al*., 2018). The administration of doxorubicin is limited because of its cardiotoxic side effects. The cardiotoxic resulting impact can be caused by the formation of ROS (Reactive Oxygen Species), so lipid peroxidation, calcium dysregulation, and obstacles to energy transfer can cause heart failure. The cardiotoxic effect also correlates with the formation of the primary metabolite of doxorubicin, namely doxorubicinol (Reis-Mendes *et al*., 2019). In administering the FAC regimen (5-Fluorouracil+Doxorubicin+Cyclophosphamide), it is known that the primary metabolite with the greatest cardiotoxic effect is doxorubicinol. The study was conducted on differentiated H9C2 cell media (Reis-Mendes *et al*., 2019). Doxorubicin in the body will be metabolised by the Carbonyl Reductase Enzymes (CBR1 and CBR3), forming its main metabolite, doxorubicinol. CBR3 enzymes in the body are expressed less than CBR1. Thus, the effect of CBR3 is not associated with

doxorubicin and doxorubicinol levels (Reis-Mendes *et al*., 2019).

Based on the above explanation, it is necessary to conduct further reviews of the doxorubicin-containing regimens in breast cancer patients to control the risk of cardiotoxic effects that can arise. The main articles to be reviewed were written by Wenningmann et al. (2019), Hanna et al. (2014) regarding the cardiotoxic effects of doxorubicin and doxorubicinol, Shi et al. (2017), and Lal et al. (2008) regarding CBR1 polymorphisms and checking methods of individual genetic profiles, and Harahap et al. (2020) regarding methods of analysing levels of doxorubicin and doxorubicinol in dried blood samples. The results of this review can then be a recommendation for the implementation of drug therapy monitoring in the administration of doxorubicin as breast cancer therapy. Doxorubicin and doxorubicinol levels can be analysed using biosampling techniques in the form of Dried Blood Spot (DBS) samples using ultra-high performance liquid chromatography-tandem mass spectrometry (LCMS/MS). The selection of this biosampling technique is based on the fact that the volume needed is small, the risk of bacterial contamination is small, and it is less invasive. It can be stored for long periods with almost no decrease in the analyte quality (Gupta & Mahajan, 2018; Mahajan *et al*., 2018). Furthermore, blood samples of breast cancer patients on extracted DBS can be injected into LCMS/MS triple quadrupole. LCMS/MS has high specificity and shorter detection times. In previous studies, LLOQ values of doxorubicin and doxorubicinol on DBS blood samples for doxorubicin were obtained at ten ng/ml and for doxorubicinol of four ng/ml (Harahap *et al*., 2020). DBS can also be used to evaluate the incidence of polymorphisms in CBR1 (Gonzalez-Covarrubias *et al.,* 2007).

Methods

Data sources

Data was sourced from ScienceDirect and Pubmed databases to conclude relevant studies on the role of Polymorphism CBR1 in cardiotoxicity after administering doxorubicin, which is related to its metabolite, doxorubicinol. Searches were also conducted on other points on the reference lists that fulfilled the review needs for further relevant publications. All manuscripts were checked for eligibility.

Study selection

Studies were selected by the association of polymorphism CBR1 with induced cardiotoxicity caused by the administration of doxorubicin. Studies that examined the impact of polymorphism on several genes of CBR1 with alteration of CBR1's pharmacokinetics were included. Suitable analysis was also added based on required information, such as its ability to be detected by Liquid Chromatography Tandem Mass Spectrometry with Dried Blood Spot Sampling method. The oldest year of the publication release was "*2005*" to represent this study as the updated review. Therefore, publications released before 2005 were not included. Studies that explore other specific genes on CBR1 and studies with odds ratios (ORs) or relative risks (RRs) were not specifically included to avoid any bias and difficulty with the conclusion. This study was not limited to race, age, weight, or social background.

Data extraction

Data were extracted based on the publication year, study objectives, specific gene polymorphism in CBR1, the specific pathway of cardiotoxicity, factors that alter the doxorubicin and doxorubicinol level in the blood, and the method for measuring the doxorubicin and doxorubicinol level in breast cancer patients' blood. The risk ratio written in the reference publication decided the summary.

Result

A total of 93 studies were screened through the literature search, and 57 were included in the studies for full eligibility. The other 36 were excluded as they were not in line with the aim of this study. Among the 57 studies, three Gonzalez-Covarrubias et al., 2008; Lal et al., 2008) stated that the administration of doxorubicin induced the polymorphism of CBR1 associated with the risk of cardiotoxicity. A total of 20 studies (Gonzalez-Covarrubias *et al*., 2007; Kassner *et al*., 2008; Lal *et al*., 2010; Hanna *et al*., 2014; Damiani *et al*., 2016; Aniogo *et al*., 2017; dos Santos *et al*., 2017; Gavila *et al*., 2017; Henninger & Fritz, 2017; Jo *et al*., 2017; Shi & Di, 2017; Luu *et al*., 2018; Tecza *et al*., 2018; Reis-Mendes *et al*., 2019; Wenningmann *et al*., 2019; Zeng *et al*., 2019; Zhao *et al*., 2019; Kalyanaraman, 2020) were included as support studies for the previous objective. The three main studies stated above declared four pothe lymorphism genetics at CBR1 gene both in the untranslated region and coding region (Piska e*t al*., 2017; Gonzalez-covarrubias *et al*., 2008; Lal *et al*., 2008). The occurrence of the fourth of them

caused alteration in CBR1's pharmacokinetic and interfered with the result of doxorubicin and doxorubicinol levels in the body (Gonzalez-Covarrubias *et al*., 2008; Lal *et al*., 2008; Piska e*t al*., 2017).

"*Population*", as described in the main studies, were not limited by either race (Lal et al., 2008); the sample was Asian (Gonzalez et al., 2008), sub-Saharan African, European Native American, or East Asian ancestry. One study (Harahap *et al*., 2020) stated the analysis condition to measure doxorubicin and its metabolites, doxorubicinol after treatment.

Figure 1 shows a flow chart of the selection of studies, while the complete search term used for the literature search is described in Table I.

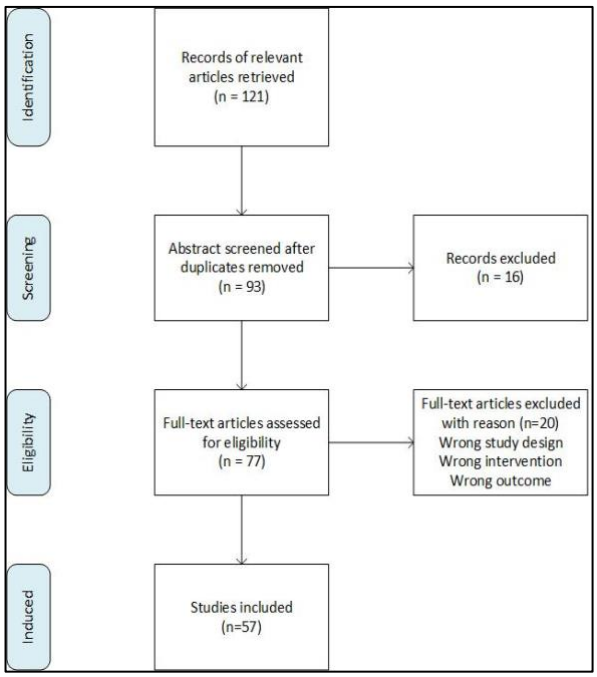

Figure 1: Flowchart of the study selection

Table I: The complete search terms used for the literature search

Subject	Search terms
Breast cancer	"*Breast cancer*", "*Breast neoplasm*", "*BRCA 1/2 mutation*"
CBR1	"*CBR1*", "*Carbonyl reductase*"
Polymorphism	"*Polymorphism CBR1*", "*CBR1 mutation*", "*SNP CBR1*", "*UTR CBR1 polymorphism*".
Doxorubicin cardiotoxicity	*"Doxorubicin Cardiotoxicity*", "*doxorubicin-induced cardiotoxicity*", "*SERCA2A doxorubicin*", "*doxorubicinol cardiotoxicity"*
DBS	"*DBS sampling method*", "*Dried Blood Spot sampling*", "*Finger prick sampling method*", "*blood perifer*".
PCR	"*PCR*", "*Polymerase Chain Reaction*", "*DNA amplification*"
LCMS/MS	"*Liquid Chromatography Tandem Mass Spectrometry*", "*Liquid Chromatography*"
Others	*"Bioanalysis"*, "*protein precipitation*", "*whole blood*", "*validation methods*"

Doxorubicin and doxorubicinol

Doxorubicin is an anthracycline class of anti-cancer drugs. Several mechanisms of action are involved in cell death by doxorubicin, namely, inhibition of topoisomerase II, intercalation with DNA, and the production of oxidative stress. Topoisomerase II is an ATP-dependent enzyme that binds to DNA and causes a double chain break by lowering the induced torque, thus enabling the process of DNA replication. Doxorubicin forms a bond with alpha and beta topoisomerase II (DOX-TOP II) (Henninger & Fritz, 2017). DOX-TOP II alpha interactions inhibit DNA replication by disrupting the cell cycle at the G1/S stage causing the cell to die. Intercalation between DNA and doxorubicin occurs in areas with many GC nitrogen bases because it has a strong hydrogen bond and causes damage to DNA (Sottani *et al*., 2013; Henninger & Fritz, 2017; Alexieva *et al*., 2018; Gupta & Mahajan, 2018; Wenningmann *et al*., 2019; Zeng *et al*., 2019).

Several pathways mediate the mechanism of intracellular oxygen radical formation. First is the reduction of the electron doxorubicin which is catalysed by the cytochrome P450 reductase enzyme to form semiquinone radicals that cause the formation of superoxide molecules. Superoxide can form hydrogen peroxide which is very reactive and destructive (Henninger & Fritz, 2017). Secondly, the interaction between doxorubicin and iron forms a doxorubicin-iron complex that can react with hydrogen peroxide and form hydroxyl radicals that break the DNA strand and cause apoptosis (Henninger & Fritz, 2017). In the mitochondria, the semiquinone fraction of doxorubicin reduces oxygen, thereby increasing oxygen radical production, which causes mitochondrial dysfunction (Henninger & Fritz, 2017). Damage to the mitochondria can cause a reduction in adenosine triphosphate (ATP). Increased doxorubicin accumulation in the mitochondria is found in the heart tissues which requires 20-40% of energy from the cellular volume, thus, making the heart tissue very vulnerable to oxidative damage mediated by doxorubicin doxorubicin (Davani *et al*., 2005; Rasola & Bernardi, 2011; Damiani *et al*., 2016; Mitry & Edwards, 2016; Alexieva *et al*., 2018; Gunawan *et al*., 2018; Wenningmann *et al*., 2019; Reis-Mendes *et al*., 2019).

In the body, doxorubicin will be catalysed by the enzyme Aldo/keto reductase (AKR) and carbonyl reductase enzymes (CBR1 and CBR3) to form the main metabolite, namely doxorubicinol by reducing two electrons with cofactor dependent NADPH (Reis-Mendes *et al*., 2019). Besides doxorubicin, doxorubicinol also affects Ca^{2+} ions systolic. Doxorubicinol affects the function of SERCA2A (sarcoma/endoplasmic reticulum Ca^{2+} -ATPase), this protein acts as an ion receptor and pump which functions in the regulation of calcium ion transfer between the cytoplasm and sarcoplasmic reticulum (Wenningmann *et al*., 2019). This, in turn, can disrupt the systolic calcium ion levels triggered by doxorubicinol. This disorder can then trigger apoptosis associated with calcineurin, which activates nuclear factors in activated T-lymphocytes and causes cells to die (Malatkova *et al*., 2010; Mitry & Edwards 2016; Jo *et al*., 2017; Momenimovahed & Salehiniya, 2019; Kalyanaraman, 2020).

CBR1

CBR1 consists of 277 amino acids and has a molecular weight of 30,375 Da. CBR1 contains many GC bonds that extend from the first exon. The concentration of this enzyme is high in tissues that have close contact with exogenous compounds, such as the liver, digestive tract epithelial cells, and epidermis. Polymorphisms against CBR1 are thought to play a role in the various activities and expressions of CBR1. Research by Jo and colleagues in 2017 showed that inhibition of the enzyme CBR1 can improve the efficacy of chemotherapy containing doxorubicin in its treatment regimen (Malatkova *et al*., 2010; Jo *et al*., 2017; Piska *et al*., 2017; Tecza *et al*., 2018).

Dried blood spot

Dried Blood Spot (DBS) collects blood drops, starting from the tip of a finger and dripping whole blood directly on filter paper. Blood sampling using DBS can be used for quantitative analysis using ultra-high-performance liquid chromatography-tandem mass spectrometry. DBS applications range from HIV-1N diagnoses to pharmacokinetic and toxicological studies. The sample size is generally 10-20 µl (Kadjo *et al*., 2016; Leeman *et al*., 2018).

The use of fingerprick biosampling in the form of DBS is more beneficial than the use of venipuncture caused by several factors, namely:
1) Required less blood volume
2) Non-invasive
3) More comfortable for patients
4) Easier logistics
5) Simpler storage for retrospective analysis
6) Fewer labor qualifications
7) Little bacterial contamination

In addition to its use in quantitative analysis, DBS can also be used in qualitative analysis in determining the DNA of species analysed using Polymerase Chain Reaction (PCR) (Grüner *et al*., 2015; Lim, 2018; Mahajan *et al*., 2018; Gupta & Mahajan, 2018).

LCMS/MS

Ultra-high-performance liquid chromatography systems generally have the same principles as HPLC. The difference lies in the particle size in the column, which reaches less than 2.0 micrometres, and the resulting pressure reaches 15,000 psi. Hence, the efficiency of separating the samples in the column becomes better, and the sensitivity increases. The smaller the particle size in the column, the shorter the flow of the analyte diffusion, so the time needed for analysis on ultra-high-performance liquid chromatography is shorter (Harmita *et al*., 2019; Mirzaei & Carrasco, 2016; Emara *et al*., 2015; Milman, 2015; Kang, 2012; Banerjee & Mazumdar, 2012).

Polymerised Chain Reaction (PCR)

PCR is an enzymatic method for DNA amplification, i.e. the replication of specific DNA sequences *in vitro*. This technique can be used for samples containing very few DNA molecules to be amplified exponentially several times. PCR is generally carried out in 10-200 microlitre volumes in small test tubes measuring 0.2 - 0.5 ml in a thermal cycler. PCR allows the acquisition of large copies of DNA fragments from extracted DNA. DNA synthesis using PCR consists of 20 to 35 cycles, each consisting of three stages: denaturation, annealing, and elongation (Rahman *et al*., 2013; Kadri, 2019).

Discussion

Cardiotoxicity in the administration of chemotherapy is defined as a reduction in Left Ventrice Ejection Fraction (LVEF) by 5% to 55% with symptoms of heart failure and a reduction in LVEF by 10% to 55% without symptoms of heart failure (Gavila *et al*., 2017). LVEF is the amount of blood released by the left ventricle per heartbeat. The administration of doxorubicin is limited by the cardiotoxic effects that are believed to occur after administration. Based on research conducted by Wenningmann and colleagues, both doxorubicin and doxorubicinol have activity in triggering cardiotoxic effects in breast cancer patients (Wenningmann *et al*., 2019). The main mechanism of doxorubicin believed to cause cardiotoxic effects, is oxidative stress, which

causes lipid peroxidation and damage to the mitochondria, which ends in cell apoptosis and cardiotoxicity (Damiani *et al*., 2016). As for doxorubicinol, it has a mechanism of interference with SERCA2A (Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase). This disrupts the calcium levels in the cytosol and causes cell apoptosis Piska *et al*., 2017). Doxorubicinol is more potent in calcium dysregulation. Both mechanisms are summarised in Figure 2.

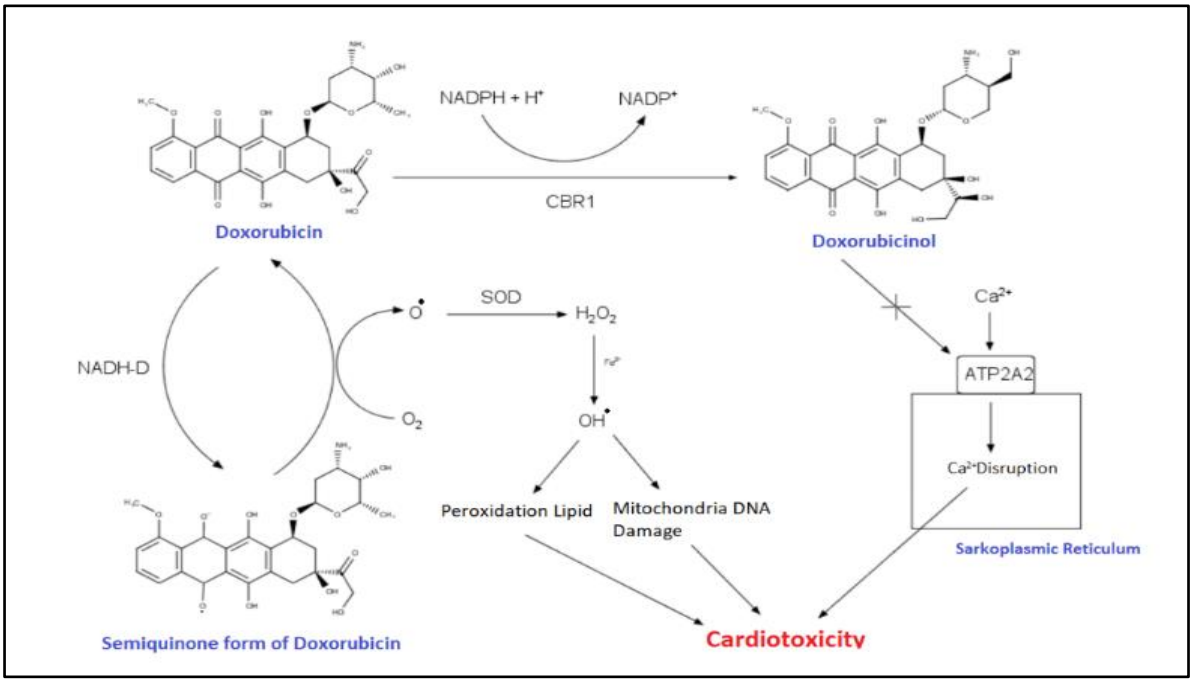

Figure 1: Cardiotoxicity effect caused by doxorubicin and doxorubicinol pathway. Doxorubicin is reduced into its semiquinone form automatically in the presence of NADH-Dehydrogenase. Thus, the semiquinone form is unstable, transfers its electron to oxygen, and forms a superoxide anion. In the body, SOD (Superoxide Dismutase) converts the superoxide anion to hydrogen peroxide. The Fenton system converts hydrogen peroxide to a hydroxyl radical, which causes cardiotoxicity. On the other hand, doxorubicin with cofactor NADPH+H+ is changed to doxorubicinol by CBR1. Doxorubicinol inference SERCA2A (Sarco / Endoplasmic Reticulum Ca^{2+}-ATPase) functions and causes Ca^{2+} disruption, soon leading to cardiotoxicity. (Source: Aniogo *et al*., 2017; Momenimovahed & Salehiniya, 2019; Wenningmann *et al*., 2019; Kalyanaraman, 2020)

In rabbits with isolated heart muscle, doxorubicinol reduces systolic myocardial function 30 times more potently than doxorubicin. It was also found that doxorubicinol, with a concentration 100 times lower than doxorubicin, can increase calcium release from the sarcoplasmic reticulum by three to fifteen times more potent than doxorubicin (Hanna *et al*., 2014). Also, doxorubicinol was found in high concentrations in the heart, which indicates that the accumulation of doxorubicinol is also in the heart (Mitry & Edwards, 2016).

Based on the explanation above, the concentration of doxorubicin in the body causes the risk of cardiotoxicity due to its accumulation in the heart. The formation of the secondary alcohol metabolite doxorubicin, doxorubicinol, increases the risk of cardiotoxicity because of its more potent nature in SERCA2A. It aggravates the dysregulation of calcium ions, which previously occurred after administration of doxorubicin. Therefore, higher doxorubicinol levels can increase the risk of cardiotoxicity.

The percentage of cardiotoxic risk studied in 25 breast cancer patients in Indonesia by Harahap and colleagues in 2020 is less than 4%, with a cumulative dose of doxorubicin in the range of 49.11 mg/m^2 - 303.70 mg/m^2. This conclusion is based on the research conducted by Luu and colleagues in 2018, which stated that the percentage of cardiomyopathy risk was 4% at doses of 500-550 mg/m^2, 18% at doses of 551-600 mg/m^2, and 36% at doses > 600 mg/m^2 (all cumulative doses). Although the percentage of cardiotoxic risk in Indonesia is relatively low, the implementation of Monitoring Drug Therapy still needs to be done because the cardiotoxic effects can be viewed from

other aspects besides the cumulative dose after administration of doxorubicin to obtain effective treatment (Luu *et al*., 2018).

Xenobiotic compounds induce expression of carbonyl reductase 1 (CBR1) through the process of the xenobiotic responsive element (XRE), thereforeCBR1 concentration describes the induction status of individuals by xenobiotic compounds that describe the concentration of the xenobiotic compounds (Malatkova *et al*., 2010). The highest concentration of the carbonyl reductase enzyme is in the liver, followed by the kidneys and digestive tract tissues Kassner *et al*., 2008). One xenobiotic compound catalysed by CBR1 is doxorubicin. CBR1 is the main enzyme that catalyses the reduction of two-electron doxorubicin into its main metabolite, namely doxorubicinol with the help of NADPH as a co-factor. (Gonzalez-Covarrubias *et al*., 2007; Lal *et al*., 2008; Gonzalez-covarrubias *et al*., 2008; Lal *et al*., 2010; Piska *et al*., 2017).

Doxorubicinol concentration is not the same in the plasma after administering the same dose of doxorubicin in each breast cancer patient. This can be caused by variability in the genetic makeup of individuals in expressing and regulating CBR1 activity (Kassner *et al*., 2008). The events of CBR1 variability are associated with the polymorphism of the CBR1 coding gene. Two forms of polymorphism found in the CBR1 enzyme affect the pharmacokinetic profile of doxorubicin, i.e., in 3'UTR and the coding region. The polymorphisms in CBR1 are summarised in Table II.

Table II: The occurrence of CBR1 polymorphisms and their effects on doxorubicin and doxorubicin

No	Polymorphism	Location	Wild type	Variant Type	Pharmacokinetic profile changes
1	+967 G > A	3'UTR	+967GG	+967GA	Decreased doxorubicin clearance; increased exposure to doxorubicin
2	262 G > A	Exon 1	262GG	262GA	Decreased doxorubicin V_{max}; and decreased catalyst efficiency (K_{cat}/K_m)
3	1096 G > A	3'UTR	1096GG	1096GA; 1096AA	Decreased doxorubicin clearance; increased doxorubicin exposure; decreased CBR1 activity; decreased mRNA CBR1 level
4	627 C > T	Exon 3	627CC	627CT; 627TT	Decreased doxorubicin clearance; increased exposure to doxorubicin

Sources: Gonzalez-covarrubias *et al*., 2008; Lal *et al*., 2008b; Piska *et al*., 2017

The incidence of SNPs at 1096 G > A, 967 G > A, and 627 C > T experienced similar changes, such as decreased clearance of doxorubicin and increased exposure to it. This decreased clearance is associated with decreased activity of CBR1, a major enzyme in the metabolic process of doxorubicin (Lal *et al*., 2010; Shi *et al*., 2017). This indicates a decrease in the conversion of doxorubicin to doxorubicinol.

Single Nucleotide Polymorphism (SNP) at 262 G > A causes changes in the amino acid- valine to isoleucine resulting in a decrease in the *Vmax* after the administration of doxorubicin. *Vmax* describes the maximum elimination rate, which, in this case, occurs in doxorubicin. The decrease in *Vmax* is associated with a decrease in the elimination of doxorubicin, therefore, doxorubicin is reduced by metabolic processes. Thus, the formation of doxorubicinol also decreases when polymorphisms occur at 262 G> A (Malatkova *et al*., 2010; Shargel & Yu, 2012; Shi & Di, 2017).

A polymorphism at 1096 G > A results in decreased CBR1 expression. So, it can be correlated with the process of converting doxorubicin to doxorubicinol by CBR1 reduced. Thus, doxorubicinol levels are reduced (Lal *et al*., 2008; Gonzalez-covarrubias *et al*., 2008; Shi & Di, 2017).Based on the data and explanation above, the incidence of CBR1 polymorphisms at 3'UTR and the coding region affects the levels of doxorubicin and doxorubicinol. The polymorphism in CBR1 decreases the change of doxorubicin to doxorubicinol. Thus, doxorubicinol levels decrease after the administration of doxorubicin to individuals with a CBR1 polymorphism profile.

Most CBR1 polymorphisms transition from guanine to adenine, indicating that patients with adenine alleles at certain amino acid positions can reduce the cardiotoxic effects after the administration of doxorubicin. Based on some previous studies, there is no specific relationship between the incidence of polymorphism towards ethnicity (Shi & Di, 2017). Research conducted by Gonzalez-Covarrubias and colleagues in 2008 with donor samples of black and white populations did not provide any significant difference in the concentration of expression.

Based on the explanation above, there is a connection between CBR1 polymorphisms and cardiotoxic effects after the administration of doxorubicin. The occurrence of CBR1 polymorphisms affects levels of doxorubicinol in the body. In the heart, it becomes lower (Gonzalez-

Covarrubias *et al*., 2008; Lal *et al*., 2008; Piska *et al*., 2017). Meanwhile, low doxorubicinol levels can reduce the risk of cardiotoxicity in breast cancer patients. Thus, patients with a genetic profile of CBR1 polymorphisms tend to lower the risk of cardiotoxicity.

Several researchers have conducted studies on quantitative analysis of doxorubicin and doxorubicinol in breast cancer patients. So the methods that the author can choose vary. The author's method is developed by Harahap and colleagues in 2020.

Blood sampling was performed by Harahap and colleagues in 2020 using the DBS fingerprick biosampling technique. This biosampling technique is very advantageous compared to blood drawn from veins (venipuncture) conducted by Sottani and colleagues in 2013. Blood collection using DBS has several advantages: the volume required is small (10-20 µl), patients feel more comfortable when taking blood, better stability of analytes, and easier storage of DBS (Sottani *et al*., 2013). Although the results of blood volume optimisation on DBS conducted by Harahap and colleagues in 2020 were more than 10 µl compared to what is stated in the literature, which is as much as 30 µl. Compared with the thesis research conducted by Puspitasari in 2020, the volume was relatively small because the retrieval with venipuncture requires 3 ml of whole blood for each patient.

The standard used in research conducted by Harahap and colleagues in 2020 is Hexamethylphosphoramide (HMPA). This selection was based on HMPA, which can be analysed under the same conditions as doxorubicin and doxorubicinol, that is, with a positive ESI mode because HMPA is alkaline-based by its structure, which has a tertiary amine group (R3N) that can be protonated through the addition of H^+ to its parent ion. This selection was also strengthened by the research conducted by Pretty and colleagues in 2012, in which the study used HMPA as a standard in the analysis of anti-neoplastic drugs, and one of the drugs analysed was doxorubicin. (Pretty *et al*., 2012; Harahap *et al*., 2020).

In developing a method by Harahap and colleagues in 2020, the extraction method used was a protein precipitation method. The solvent used to deposit protein is methanol. Methanol is also used as a solvent for analytes. This selection is based on the good solubility of doxorubicin and doxorubicinol in methanol. This method also uses aquadest to dissolve blood and analyte components from DBS paper. In a study conducted by Sottani and colleagues 2013, the sample extraction used was solid Phase Extraction (SPE). The author did not choose the SPE extraction method because it uses more reagents and a more complex process. There have not been studies that do the extraction of doxorubicin and doxorubicinol from DBS paper with SPE, so, this selection needs further review (Evans *et al*., 2009; Moein *et al*., 2017; Kaza *et al*., 2019).

Samples and standards that have been extracted are injected into the LCMS/MS instrument, with selected analysis conditions in the form of:

1) The UPQ BEH C18 Acquity column (2.1 x 100 mm; 1.7 µm); 2) The mobile phase is 0.1% acetic acid and acetonitrile; 3) The modes of detection were positive ESI and MRM with m/z values 544.22 > 397.06 for doxorubicin, m/z 546.22 > 363.05 for doxorubicinol, and 180.03 > 135.16 for hexamethylphosphoramide; 4) Gradient elution mode; 5) The flow rate of 0.15 ml/minute. This condition has been optimised by Harahap and colleagues in 2020.

These conditions obtain LLOQ values for doxorubicin at 10 ng/ml and for doxorubicinol at 4 ng/ml. The LLOQ value obtained is higher than the LLOQ value obtained in the study of Sottani and colleagues in 2013, namely for doxorubicin at 2 ng/ml and doxorubicinol at 1 ng/ml. In the study of Seemren and colleagues 2018 with an LLOQ of 1 ng/ml for doxorubicin. This can be caused by the biosampling technique used by Harahap and colleagues in 2020 in the form of DBS, where the drawn blood was capillary. The sensitivity in the DBS sample is lower than in the plasma sample, this can affect the LLOQ value (Sottani *et al*., 2013; Van De Merbel *et al*., 2014; Seemren *et al*., 2018). This statement is reinforced by the research conducted by Taylor and colleagues in 2013 which obtained different LLOQ values of acetaminophen in DBS with plasma and Cerebro-spinal fluid (CSF). The LLOQ value obtained in the DBS sample was 27.4 ng/ml, whereas in the plasma and CSF samples, the LLOQ value was 3.05 ng/ml.

This shows the effect of biosampling techniques on the value of selectivity. The difference in LLOQ values in DBS and plasma can also be influenced by drug distribution in plasma and cellular blood components, However, the LLOQ value obtained by Harahap and colleagues in 2020 has met the appropriate requirements in the EMEA guideline and the FDA guideline with a value of -15.07% to 3.94% for "*% difference*" and 9.55% for "*% CV*" in doxorubicin, and a value of -19.18% to 0.97% for "% difference" and 8.52% for "% CV" on doxorubicinol, likewise for other parameters such as linearity, accuracy, precision, selectivity, carry-over, recovery stability, matrix effect, and dilution integrity that meet the requirements of the EMEA guidelines and the FDA guideline (EMEA, 2011; Taylor *et al*., 2013; FDA, 2018; Harahap *et al*., 2020).

The development of the method that was carried out by Harahap and colleagues in 2020 has also been

applied to 25 breast cancer patients who have a doxorubicin regimen in their treatment. Thus, the authors believe the method used by Harahap and colleagues in 2020 is the best method to analyse doxorubicin and doxorubicinol levels in breast cancer patients. Based on previous research, CBR1 polymorphism analysis mostly uses the polymerase chain reaction (PCR). PCR has the advantage of high specificity and sensitivity, high repetition, and reproducibility, and is easy to do (Santos *et al*., 2017).

The selection of specimens in the form of blood was carried out following several studies that had been carried out previously, such as those conducted by Lal and colleagues in 2008, and Gonzalez-covetarrubias and colleagues in 2008. Researchers chose the DBS biosampling method in CBR1 polymorphism analysis for several reasons similar to the selection of DBS as a biosampling technique in the analysis of doxorubicin and doxorubicinol levels in breast cancer patients. This use is further strengthened by the research conducted by Mahajan and colleagues in 2018, which compared the results of the hepatitis C virus RNA genotype analysis in plasma and DBS samples. This study showed 100% success in obtaining RNA genotypes in all samples (n = 162) in both plasma and DBS samples. So DBS can be used for genotype analysis in this review (Gonzalez-Covarrubias *et al*., 2008; Gupta & Mahajan, 2018)

The DNA extraction protocol from DBS samples can follow the protocol listed in QIAamp® DNA Mini and Blood Mini Handbook (2016) (QIAGEN, 2016). The primary selection used to conduct CBR1 genotyping is F-5'-GAGGGGTAGGGATGGTTCAG-3', R-5'-CCTAGGGAGGCGTTATGGAC-3' for exon 1, and F-5'-TCACCTCTCTACGGGATTGTT-3', R-5'-TCCCTTGACCTTTTAGGTTGA-3' (Lal *et al*., 2008). After obtaining the amplified DNA, the sample needs to be checked for sequences using Capillary Electrophoresis (CE) (Karger & Guttman, 2009)). Thus, DNA sequence samples are obtained and can be compared with CBR1 DNA sequences in their natural form.

Conclusions

Higher levels of doxorubicinol, increasing the risk of cardiotoxicity after administration of doxorubicin, are associated with more potent properties in deregulating calcium ions in the cytoplasm. Polymorphism in CBR1 (967 G > A; 262 G > A; 1096 G > A; 627 C > T) changes the activity and expression of CBR1 where the conversion of doxorubicin and doxorubicinol decreases so that the level of doxorubicinol decreases causing the risk of cardiotoxicity also to decrease. The most optimal method in the analysis of doxorubicin and doxorubicinol is the Dried Blood Spot blood samples using LCMS/MS with conditions such as the Acquity® UPLC BEH C18 column (2.1 x 100 mm; 1.7 µm); mobile phase of 0.1% acetic acid and acetonitrile; positive ESI detection mode and MRM with m/z values 544.22 > 397.06 for doxorubicin, m/z 546.22 > 363.05 for doxorubicinol, m/z 180.03 > 135.15 for Hexamethylphosphoramide as the internal standard; gradient elution mode; and a flow rate of 0.15 ml/minute. The extraction method used is protein precipitation using methanol.

Availability of data and materials

Clinical data for 25 breast cancer patient samples from a study done by Harahap et al. (2020) is available at (https://innovareacademics.in/journals/index.php/ijpps/article/view/36707)

Conflict of interests

The authors declare no conflict of interests.

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