ISBN: 978-628-96613-1-6. ISSN: 2414-6390. Digital Object Identifier: https://dx.doi.org/10.18687/LACCEI2025.1.1.1649

Doxorubicin-Induced Modulation of NF-κB, Bcl-2, and Bax Expression in Breast Cancer Cell Lines

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Abstract– ***Abstract– Breast cancer is the most common malignancy and one of the leading causes of death in women worldwide. Breast cells grow uncontrollably, forming tumors that can spread through the blood or lymphatic system. The treatment of breast cancer includes surgery, radiotherapy and the use of drugs such as doxorubicin, which aim to reduce the size of the tumor and improve the patient's condition. However, their efficacy is limited due to factors such as cellular resistance and efflux transporter activity. The aim of the work was to analyze the expression of NF-kB, Bcl-2 and Bax in MCF-7 and MDA-MB-231 breast cancer cell lines treated with doxorubicin (DOX). IC50 analysis of DOX was performed by MTT assay, gene quantification was by qPCR and In silico analysis of TLR4 and DOX was performed. MTT results showed that the IC50 for MCF-7 and MDA-MB-231 were 0.1µM and 0.3µM respectively. The qPCR results showed that the overexpression of NF-κB was 1.825±0.054 and 10.85±1.000 in MDA-MB-231 and MCF-7 cell lines respectively. For Bax, the expression level was 1.827±0.1036 and 0.6869±0.092 in MDA-MB-231 and MCF-7 cell line consecutively. Furthermore, In silico analysis showed that DOX docks into the extracellular domain cavity of TLR4 through interactions with Lys52 and Ile65A residues.***

Keywords-- Doxorubicin, breast cancer, NF-κB, Bax, Bcl-2.

I. INTRODUCTION

Currently, according to the global cancer observatory of the World Health Organization (WHO), the main types of neoplasia are breast, prostate, lung and colon cancer [1]. On the other hand, according to the International Agency for Research on Cancer (IARC), in 2022 breast cancer accounted for 17.7% of new cases and ranked first among incidences in many countries. Breast cancer is considered the most frequent neoplasm in women [2], [3]. Millions of women are affected by breast cancer every year, making it one of the most common and dangerous cancer diseases in the world, which is why it is considered a serious public health problem.

Cancer is a disease characterized by the uncontrolled growth and spread of abnormal cells in the body. It can affect any part of the body and can have various causes such as genetic, environmental or lifestyle factors [4].

Breast cancer originates when breast cells begin to grow in an uncontrolled manner, which involves the formation of a tumor that can be seen on an x-ray or can be felt as a mass or lump in the breast. In addition, this type of neoplasm usually spreads when the cancerous cells reach the blood circulation or the lymphatic system, thus reaching other parts of the body causing metastasis [3], [5].

The most frequent types are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), the latter representing between 5% and 15% of cases. The classification of these tumors is based on their morphology and their capacity to form tubules or ducts. On the other hand, to evaluate prognosis, the Nottingham classification system is used, where grade I tumors have a better prognosis compared to grade II and III tumors. In addition, there are other important factors such as age, tumor size, presence of hormone receptors, HER2 receptor status, Ki-67 proliferation index, lymphovascular invasion, and lymph node status [6].

Currently, there are several treatments which are related to the tumor grade, such as: surgery, radiotherapy, chemotherapy, etc. Within neoadjuvant chemotherapy, commonly used chemotherapy drugs such as doxorubicin aim to reduce tumor size and improve the patient's overall stage.

Doxorubicin (DOX), an effective inhibitor of topoisomerase II (TopII) that prevents DNA replication in cancer cells, generates free radicals and oxidative stress [7]. But their efficacy is often limited by resistance resulting from a variety of factors, including increased activity of drug efflux transporters, increased oncogenic factors and lack of targeted delivery [8]. However, research suggests that cell damage

caused by doxorubicin releases damage-associated molecular patterns (DAMPS), whereby doxorubicin would trigger a cascade of molecular events that culminate in the modulation of some receptors and the subsequent stimulation of *NF-kB*, *Bax* and *Bcl-2*, favoring resistance to cell death and therefore therapeutic failure [9].

Some research suggests that the *NF-κB* transcription factor pathway is a crucial regulator of inflammation and immune responses. In addition, aberrant *NF-κB* signaling has been identified in many types of cancer. Importantly, downstream of key oncogenic pathways, such as RAS, BCR-ABL and Her2; *NF-κB* regulates the transcription of target genes that promote cell survival and proliferation, inhibit apoptosis, and mediate invasion and metastasis [10].

Regarding *NF-κB* activation, Toll-like receptor 4 (TLR4) when expressed on the cell membrane, exists as a complex with the co-receptor myeloid differentiation protein-2 (MD-2), which is essential for lipopolysaccharide (LPS) recognition by the TLR4-MD-2 complex. LPS binding causes dimerization of the TLR4-MD-2 complex, resulting in the activation of downstream mediators, including the transcription factor nuclear factor transcription factor (*NF-κB*), which increases the production of proinflammatory molecules, such as tumor necrosis factor (*TNF*)-α, interleukin *IL-1β*, *IL-6*, chemokines, enzymes, and reactive oxygen and nitrogen species [11], [12].
In view of the above, the present research work was aimed at analyzing whether DOX stimulates *NF-kB* expression in breast cancer cell lines (Fig 1).

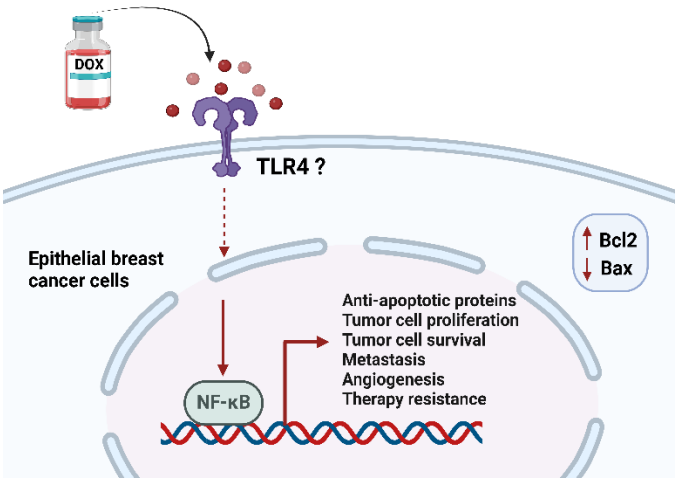


Fig 1. TLR4 signaling pathway for NF-κB activation.

II. MATERIALS AND METHODS:

A. *Cell Culture*

The breast cancer cell lines, MCF-7 and MDA-MB-231, were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic. Cells were incubated at 37 °C, 5% CO_2 until optimal confluency (> 80%) was reached for subsequent use.

B. *Determination of the IC50 de DOX*

For the determination of IC50 of doxorubicin, MCF-7 and MDA-MB-231 cells were treated with decreasing concentrations of DOX (50μM - 0.02μM). IC50 analysis was performed using the cell viability assay by the MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) [13][14].

C. *Analysis of NF-kB, Bax and Bcl-2 expression by qPCR*

MCF-7 and MDA-MB-231 cells were cultured in 6-well plates for subsequent treatment with doxorubicin (concentration equivalent to IC50) for a period of 24 hours. Once the treatment process was completed, RNA extraction was continued using the TRIzol method according to the reagent specifications [15].

The concentration and quality of RNA was determined using the spectrophotometry technique (A260 and A280), at the same time the integrity of the genetic material was evaluated by horizontal electrophoresis, then proceeded as follows:

A. *Synthesis of complementary DNA from RNA*

An abm OneScript® Reverse Transcriptase cDNA synthesis kit was used [16], following the manufacturer's recommendations.

B. *Gene amplification*

The genes of interest; *NF-kB*, Bax and Bcl-2; were evaluated using the reagent provided by Promega (GoTaq®Real-Time PCR Systems) using the real-time polymerase chain reaction (qPCR) technique. For the analysis, specific primers were used, which are detailed in Table I.

Finally, the GADPH reference gene was used for analysis and the 2^{-ddCt} method was applied to calculate gene expression by relative quantification [17]. Data obtained from amplification were processed by relative quantification in Microsoft Excel and GraphPad Prism 8.

TABLE I
PRIMER LIST

Primers	Sequence
GADPH	F: GAGAAGGCTGGGGCTCATTT R: AGTGATGGCATGGACTGTGG
Nf-kB	F: TGAACCGAAACTCTGGCAGCTG R: CATCAGCTTGCGAAAAGGAGCC
Bax	F: CCCCCGAGAGGTCTTTTTCC R: CCTTGAGCACCAGTTTGCTG
Bcl-2	F: TCTCATGCCAAGGGGGAAAC R: TCCCGGTTATCGTACCCTGT

C. In silico simulation of TLR4 and DOX

In the RCSB PDB portal, the TLR4 receptor (3FXI) was searched and downloaded in PDB format [18], [19]. Subsequently, the PockDrug portal was used to search for protein, choosing the most drugable pocket, which was pocket 0. Using the informative Chimera-Debug tool, all protein residues were removed and the most drugable pocket was placed. On the other hand, the ligand Doxorubicin (DOX) was searched in the ChEMBL database and downloaded in PDB format (OPENABEL).

Docking and molecular dynamics simulations between DOX and the TLR4 receptor were performed in the DockThor program. Finalmente, para observar la afinidad del ligando – Receptor se utilizó el programa Chimera-Debug.

III. RESULTS

The results of treatment of MCF-7 and MDA-MB-231 cells with DOX are shown below.

A. IC50 results of MCF-7 and MDA-MB-231 treated with DOX.

After treatment of MDA-MB-231 cells with decreasing concentrations of Doxorubicin (50μM - 0.*02*μM) for 24 hours via MTT assay, the IC50 was determined to be 0.3 μM, indicating that at this concentration viable cells were reduced to 50%.

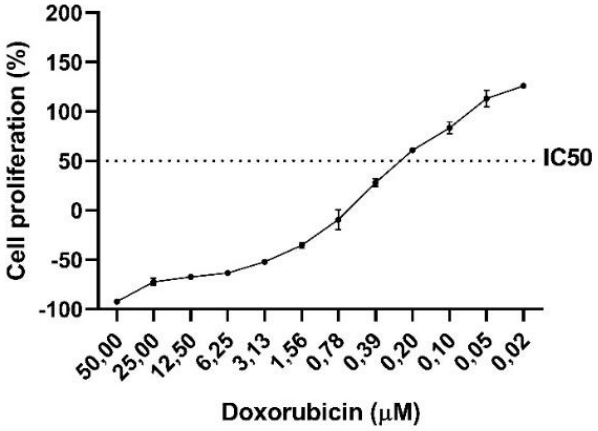


Fig. 2. Curve for the determination of the IC50 of Doxorubicin in MDA-MB-231

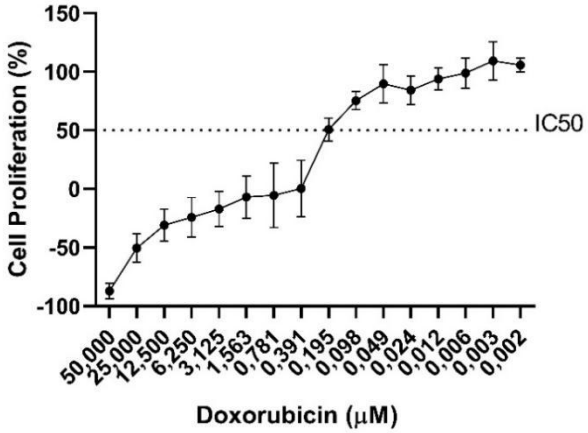


Fig. 3. Curve for the determination of the IC50 of Doxorubicin in MCF-7.

After treatment of MCF-7 cells with decreasing concentrations of Doxorubicin (50µM - 0.02µM) for 24 hours via MTT assay, it was determined that the IC50 of 0.1 µM, indicating that at that concentration viable cells were reduced to 50%.

A. Resultados de la expresión de NF-kB, Bax y Bcl-2

Fig. 3, shows the results of *NF-kB* and *Bax* expression in MCF-7 cells stimulated with DOX. Graph A, shows that MCF-7 cells express more *NF-kB* compared to the control group ($p<0.05$). Regarding the expression of Bax and Bcl-2, no expression of these genes was reported on the MCF-7 line.

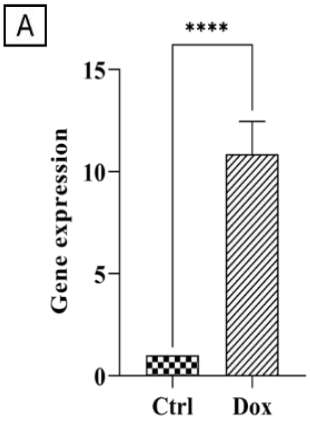


Fig. 4. Effect of doxorubicin treatment on *NF-κB* and *Bax* gene expression in breast cancer cell line MCF-7. **** represents the significant difference between experimental groups. It was considered significant when $p<0.05$. (**Ctrl**) Control and (**Dox**) Doxorubicin. (**A**) Gene expression of *NF-κB.*

Fig.5 shows the results of NF-kB and Bax expression in MDA-MB-231 cells stimulated with DOX. Graph A and B show that MDA-MB-231 cells show higher expression of *NF-kB* and Bax, respectively, when compared with the control

group ($p<0.05$). Regarding the expression of Bcl-2, no expression of this gene was reported on the MDA-MB-23 line.

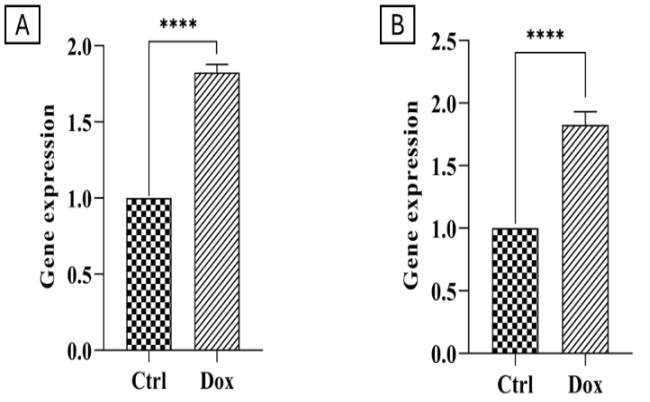
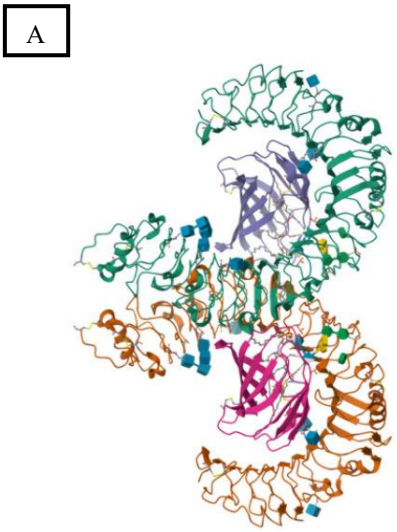


Fig. 5. Effect of doxorubicin treatment on *NF-κB* and Bax gene expression in breast cancer cell line MDA-MB-231. **** represents the significant difference between experimental groups. It was considered significant when $p<0.05$. (Ctrl) Control and (Dox) Doxorubicin. (A) Gene expression of *NF-κB* and (B) Gene expression of *Bax.*

Briefly, the qPCR results showed that the overexpression of *NF-κB* was 1.825±0.054 and 10.85±1 in MDA-MB-231 and MCF-7 cell lines respectively indicating the possible effect of DOX on the increased expression of this gene. In the case of *Bax*, the expression level was 1.827±0.1036 and 0.6869±0.092 in MDA-MB-231 and MCF-7 cell line respectively.

B. In silico Simulation Results

In the case of doxorubicin, the binding to the TLR4 protein is through an amino acid LIG2. and a hydrogen bridge bond of 1.65 Å, so there is a higher interaction.



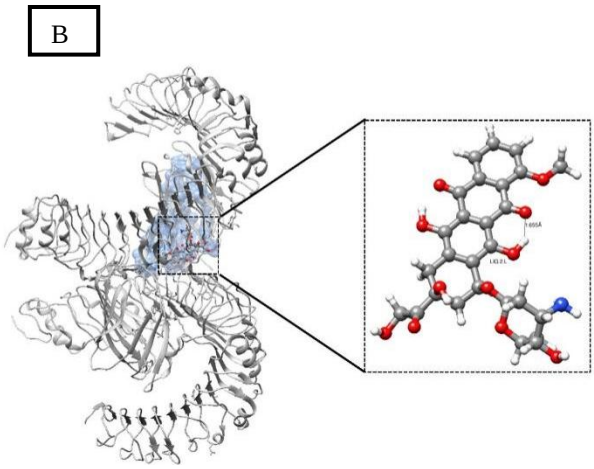


Fig 6. Molecular docking results of TLR4 (PDB ID: 3FXI) with the drugs doxorubicin. (A) Docking model of TLR4 and doxorubicin with a score or affinity of -10,454 kcal/mol. **Source:** Chimera-Debug.

Molecular docking analysis between TLR4 and DOX evidences a stable interaction with a binding energy of -10,454 kcal/mol, suggesting a high affinity of DOX for TLR4. Furthermore, computational simulation shows that DOX docks into the cavity of the extracellular domain of TLR4 through interactions with Lys52 and Ile65A residues.

These interactions may involve hydrogen bonds and Van der Waals forces, contributing to the stability of the complex. On the other hand, the identification of this binding site suggests that doxorubicin may modulate TLR4 signaling, which could have implications for the innate immune response and adverse effects associated with chemotherapy.

IV. DISCUSSION

NF-κB is one of the important transcription factors in the regulation of inflammatory signaling pathways. It has been shown that this gene can influence cell survival and proliferation, which impacts tumor microenvironment and pathogenesis. In breast cancer, *NF-κB* drives the development of cancer stem cells, promoting tumorogenesis and resistance to chemotherapeutic treatment [20].

The results of the analysis of *NF-κB* expression in breast cancer cell lines showed that DOX stimulates the expression of this gene in both MCF-7 and MDA-MB-231 cells. These results are related to those published by Devanaboyina *et al.* where it is mentioned that anthracycline chemotherapies, such as doxorubicin, can activate the transcription factor *NF-κB* and therefore favor subsequent pro-survival and chemoresistance effects [20], [21]. On the other hand, Marinello *et al.* describe a study on the prevention by metformin of doxorubicin resistance in MCF-7 and MDA-MB-23 cell lines, in that study it is mentioned that *NF-κβ* activation is established due to the induction of DNA damage by DOX, which activates the transcription factor, impairs the cytotoxicity of the drug and favors the activation of anti-apoptotic and pro-survival genes

[22]. Therefore, the results of *NF-κβ* expression in MCF-7 and MDA-MB-231 cells could suggest that DOX, in addition to fulfilling its function as a chemotherapeutic, would be associated with tumor survival, cell proliferation and chemoresistance.

The *Bcl-2* protein integrates a protein family that oversees the integrity of the genome in the cell, acting as an antiapoptotic agent. On the other hand, the Bax protein (proapoptotic), associates with other proteins for the formation of pores within the mitochondrial membrane, resulting in the release of cytochrome C which activates the caspases cascade and leads to apoptosis [23]. Thus, *Bcl-2/Bax* ratio greater than 1 represents an increased tendency for apoptotic resistance.

In our results, the simultaneous expression of *NF-κB* and Bax in DOX-treated MDA-MB-231 cells suggests that the cell is in a state of stress where it could decide between survival or death. However, the *NF-κB*/Bax ratio < 1 suggests that apoptosis is dominating over cell survival, indicating a good response to treatment. Quite the opposite, occurs in DOX-treated MCF-7 cells where the non-expression of *Bcl-2* or *Bax*, suggest that the cell may be opting for alternate mechanism such as autophagy, necroptosis or senescence, however, the expression of *NF-κB* (without induction of *Bax* or *Bcl-2*) may mean that the cell is surviving treatment by alternative mechanisms, such as activation of pro-survival and anti-apoptotic genes other than *Bcl-2*.

Aligned with our results, Campbell *et al*. in their research work targeting *Bcl-2*-regulated apoptosis in cancer, mention that the balance between pro- and anti-apoptotic *Bcl-2* proteins ensures proper regulation of programmed cell death and that, when this relationship is imbalanced, the BCL-2 family may act as a barrier to apoptosis and facilitate tumor development and resistance to cancer therapy [24]. For the above mentioned, it is suggested to perform the study of other molecular markers such as *Ki67*, CXCR4 or the Receptor related to *NF-κB* stimulation such as TLR4.

Avagimyan *et al*. revealed that doxorubicin contributes to cardiotoxicity by interacting with the TLR4 receptor of cardiomyocytes. Under this logic, and with the results of the molecular docking analysis between TLR4 and DOX evidencing a stable interaction and a high affinity of DOX for TLR4, presumably DOX could contribute positively in the molecular signaling cascade that would activate NF-kB. *NF-kB*

This study provides relevant information for the characterization of new therapeutic targets and for the design of pharmacological strategies to optimize the safety and immunomodulation profile of doxorubicin in oncology patients.

V. CONCLUSIONS

DOX stimulates NF-κB expression in MCF-7 and MDA-MB-231 cells. These results could suggest that DOX, in addition to fulfilling its function as a chemotherapeutic, is associated with tumor survival, cell proliferation and chemoresistance. The simultaneous expression of NF-κB and Bax in DOX-treated MDA-MB-231 cells and the NF-κB/Bax ratio < 1 suggests that apoptosis is dominating over cell survival, indicating a good response to treatment. The opposite occurs in DOX-treated MCF-7 cells where the non-expression of Bcl-2 and Bax suggests that the cell may be opting for alternative mechanisms, and NF-κB expression may mean that the MCF-7 cell is surviving the treatment by alternative mechanisms such as activation of pro-survival and anti-apoptotic genes other than Bcl-2. Finally, *In silico* analysis showed that DOX docks into the extracellular domain cavity of TLR4 through interactions with Lys52 and Ile65A residues.

ACKNOWLEDGMENT

This research was funded by the Universidad Católica de Santa María (UCSM) – Peru.

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