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Potential Immuno-therapeutic Role of Gold Nanoparticles on Toll-like Receptor Modulation in SKBR-3 Breast Cancer Cells

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ABSTRACT

Breast cancer is the most prevalent malignancy among women worldwide and is associated with high mortality rates in its metastatic stages. Emerging evidence highlights the role of Toll-like receptors (TLRs) in tumor progression, immune modulation, and chronic inflammation. Breast cancer remains the most commonly diagnosed malignancy among women globally and is a leading cause of cancer-related mortality, particularly in advanced metastatic stages. The complexity of breast cancer progression is increasingly attributed not only to genetic and hormonal factors but also to dysregulation of innate immune signaling pathways. Among these, (TLRs) Toll-like receptors are the class of pattern-recognition receptors, gained attention for their dual role in tumor promotion and immune activation. Recent studies have proposed TLRs as promising targets for therapeutic modulation, especially for tumor microenvironment remodeling. Gold nanoparticles (AuNPs) have emerged as highly versatile Nano-materials in cancer Nano-medicine due to their biocompatibility, tunable surface chemistry, and potential for drug delivery and immunomodulation. However, the interaction between AuNPs and TLR-mediated signaling pathways in breast cancer cells remains under-explored. This study aimed to investigate the impact of AuNPs on the expression patterns of key TLR genes in breast cancer cell line SKBR3, compared with the non-tumorigenic mammary epithelial cell line CRL-8798. Following treatment with citrate-stabilized AuNPs, total RNA was extracted and subject to qRT-PCR for the quantification of TLR gene-expression. Our results revealed that exposure to AuNPs significantly down-regulated the expression of TLR3, TLR8 and TLR9 in SKBR3 cells, suggesting the suppression of specific antiviral and pro-inflammatory pathways. Conversely, TLR1, TLR2 and TLR5 were markedly up-regulated, indicating a shift toward bacterial pattern recognition and potentially altered immune signaling dynamics. Interestingly, AuNP treatment also resulted in the up-regulation of p53, a pivotal tumor suppressor involved in apoptosis and cell cycle regulation, alongside the down-regulation of NF-κ B, key transcription factor associated with inflammation, proliferation, and cancer progression. This reciprocal regulation suggests a possible crosstalk between the p53 and NF-κ B pathways mediated by AuNP exposure. This comprehensive study demonstrates that gold nanoparticles can selectively modulate TLR expression and key oncogenic signaling pathways in breast cancer cells. These findings provide valuable insights into the potential of AuNPs as immuno-modulatory agents capable of influencing tumor-associated signaling cascades. These results will be helpful for future investigations into the therapeutic applications of nanoparticles in modulating innate immune responses in breast cancer and other malignancies.

INTRODUCTION

Breast cancer persists as a major global health burden, affecting millions of lives worldwide, and contributing to approximately 2.3 million new cases and 685,000 deaths each year (Sung et al., 2021). Among its molecular classifications, HER2-positive breast cancer constitutes about 15–20% of cases and is known for its aggressive phenotype, high metastatic potential, and resistance to standard treatments (Waks & Winer, 2019). The SKBR3 cell line, widely recognized as an in vitro model of HER2+ breast cancer, exhibits amplified HER2/neu oncogene expression and is extensively utilized for developing and testing emerging therapeutic strategies (Neve et al., 2006). Despite progress in targeted monoclonal antibody therapies such as trastuzumab, the development of intrinsic and acquired resistance remains a significant challenge, highlighting the urgent need for novel therapeutic approaches (Pondé, Brandão, & Piccart, 2019). Immunotherapy has revolutionized cancer treatment by harnessing the immune system to eliminate malignant cells (Ribas & Wolchok, 2018). A pivotal aspect of this strategy involves targeting innate immune receptors, particularly Toll-like receptors (TLRs), which serve as a critical link between innate and adaptive immune responses. TLRs function as pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), initiating downstream signaling cascades such as those involving NF-κB and IRFs which drive cytokine secretion, antigen presentation, and T-cell activation (Kawai & Akira, 2010). In breast cancer, TLRs have context-dependent roles; while receptors such as TLR3 and TLR7/8 are implicated in anti-tumor responses, others like TLR4 are associated with tumor progression, metastasis, and immune evasion (Gonzalez-Reyes et al., 2010; Salaun et al., 2006). For example, TLR4 activation in SKBR3 cells promotes pro-inflammatory cytokines such as IL-6 and TNF-α; however, sustained activation may contribute to chemotherapy resistance (Rajput et al., 2013). Therefore, finely tuned modulation of TLR activity holds potential for therapeutic benefit. Nanotechnology offers a transformative platform for precision immunotherapy. Gold nanoparticles (GNPs), in particular, are advantageous due to their adjustable size (1–100 nm), facile surface modification, biocompatibility, and photothermal capabilities (Dreaden et al., 2012). Their large surface area-to-volume ratio facilitates effective drug loading, and their chemically inert core reduces cytotoxicity risks (Murphy et al., 2008). Importantly, GNPs can inherently modulate immune pathways, acting as agonists or antagonists of TLRs depending on parameters like size, shape, coating, and dose (Zhang et al., 2018). For instance, citrate-capped GNPs have been shown to suppress TLR9 activity in dendritic cells (Bhattacharya et al., 2016), while GNPs coated with polysaccharides activate TLR4 and promote NF-κB signaling (Niidome et al., 2006). In breast cancer, GNPs have demonstrated promise in enhancing drug delivery, radio-sensitization, and thermal ablation; however, their role in directly modulating TLR pathways within HER2+ cancer cells remain insufficiently explored (Hainfeld et al., 2011; Retif et al., 2015). Preliminary findings suggest that GNP-induced modulation of TLR signaling has therapeutic potential in oncology. In melanoma models, GNPs conjugated with CpG oligonucleotides (TLR9 ligands) have been shown to enhance dendritic cell maturation and activate cytotoxic T-cell responses (Schuller et al., 2011). Likewise, TLR7 agonist-functionalized GNPs have been reported to inhibit tumor progression in lung adenocarcinoma by promoting M1 macrophage polarization (Rodrigues et al., 2018). In breast cancer models,

unmodified GNPs trigger apoptosis in MCF-7 cells through oxidative stress mechanisms (Arvizo et al., 2013), but their immune-modulatory effects in HER2+ systems are not yet well defined. SKBR3 cells, which express TLR2, TLR4, and TLR9, present a suitable model for studying GNP-TLR interactions (Ilvesaro et al., 2007). Notably, TLR3 activation in these cells initiates apoptosis via TRIF-dependent mechanisms, suggesting an exploitable therapeutic window for GNP-mediated TLR engagement (Salaun et al., 2006). Beyond direct tumor cytotoxicity, GNP-TLR interactions could reprogram the tumor immune microenvironment (TME). For example, GNPs may reverse immune suppression by reorienting tumor-associated macrophages toward a pro-inflammatory M1 phenotype, diminishing regulatory T cell (Treg) activity, or stimulating natural killer (NK) cells (Tao et al., 2017). Additionally, activating TLR pathways through GNPs may synergize with immune checkpoint inhibitors by fostering a more immunogenic TME (Sato-Kaneko et al., 2017). However, inconsistencies in outcomes across studies underscore the importance of nanoparticle design. Smaller GNPs (≤20 nm) tend to activate TLR7/8, while larger particles (≥40 nm) more effectively engage TLR4 (Tao et al., 2015). Surface modifications also influence biological interactions PEGylation reduces immune detection and clearance, whereas chitosan functionalization improves cellular uptake (Alkilany & Murphy, 2010). Thus, tailoring GNP physicochemical features is vital for achieving specific TLR modulation. Despite encouraging preclinical insights, significant knowledge gaps remain. Most research has focused on GNP interactions with immune cells, often neglecting their effects on TLR expression and signaling in tumor cells themselves. Specifically, how GNPs influence TLR networks in HER2+ breast cancer, particularly in SKBR3 cells, is not well characterized. Furthermore, the combined impact of GNP-mediated TLR activation with existing therapies like trastuzumab requires further study. To bridge these gaps, the present research investigates the immunotherapeutic potential of GNPs to modulate TLR signaling in SKBR3 cells. We hypothesize that GNPs with controlled physicochemical properties can selectively influence TLR expression and activity, modulate cytokine secretion, promote immunogenic cell death, and enhance susceptibility to immune-mediated eradication.

MATERIALS AND METHODS

PREPARATION OF AUNPS: AuNPs were manufactured through antibiotic-drug and then were characterized by several techniques like UV Vis, FTIR and XRD. Finally, they were applied on SKBR-3 breast cancer & CRL-8798 normal breast cells.

CELL CULTURE AND NANOPARTICLE TREATMENT

Human breast adenocarcinoma cell line SKBR3 and the immortalized, non-tumorigenic mammary epithelial cell line CRL-8798 (both sourced from LGC Standards, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA). The medium was supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and an antibiotic–antimycotic mixture containing 100 U/mL Penicilline and 100 µ g/mL Streptomycin (Sigma-Aldrich, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 . Upon 70–80% confluency, cells were treated with gold nanoparticles (AuNPs) at predetermined concentrations. Following 24-hour incubation period, cells were harvested for subsequent molecular analyses.

RNA ISOLATION AND QUANTITATIVE GENE EXPRESSION ANALYSIS

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany). RNA purity and concentration were assessed spectro-photometrically (A260/A280 ratio ≥ 1.8), and integrity was confirmed by gel electrophoresis. For cDNA synthesis 1 μg of total RNA was used with the QuantiTect Reverse Transcription Kit (Qiagen, Germany), according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green on the Rotor-Gene 6000 thermocycler (Corbett Research, Australia). Gene targets included Toll-like receptors (TLR1, TLR2, TLR3, TLR5, TLR8, TLR9) the tumor suppressor gene TP53, and the pro-inflammatory transcription factor NF-κ B. β-Actin served as house-keeping gene. $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative gene expression.

STATISTICAL ANALYSIS: Corbet Rotor-Gene software was used to analyze the collected data. Fold change ($2^{-\Delta\Delta Ct}$) value among 0.1- 0.5 were considered as significantly down-regulated while the value >2.0 was considered as significantly up-regulated. The expression level of TLRs, p53 and NFkB of (SKBR3) Breast-cancer cell line & (CRL-8798) immortal breast-epithelial cell lines were compared.

SYNTHESIS AND CHARACTERIZATION OF ANTIBIOTIC-CAPPED GOLD NANOPARTICLES (AUNPS)

SYNTHESIS PROTOCOL

REDUCTION METHOD: AuNPs were synthesized using an undisclosed antibiotic drug as both reducing and stabilizing agent. A 1 mM $HAuCl_4$ solution was heated to boiling under reflux. The antibiotic drug (concentration optimized for nucleation) was rapidly injected, and the reaction continued until a stable ruby-red color appeared (indicating spherical nanoparticle formation). AuNPs Crude was centrifuged at 15,000 rpm for 20-minutes and were **3**×-washed with deionized water & resuspended in sterile PBS.

CHARACTERIZATION TECHNIQUES

UV-Vis Spectroscopy (Shimadzu UV-2600) was employed which confirmed plasmon resonance peak at 520–535 nm. FTIR (PerkinElmer Spectrum Two) Identified antibiotic functional groups (e.g., amine/carboxyl stretches) bound to AuNPs. XRD (Bruker D8 Advance) Scanned 2θ range 20°–80° (Cu Kα radiation, λ = 1.54 Å). Crystallite size was calculated via Debye-Scherrer equation.

CELL CULTURE AND TREATMENT

CELL LINES

Breast cancer cell line (SKBR3) (ATCC® HTB-30™) and Immortal mammary epithelial CRL-8798 (ATCC® CRL-8798™) were used in this study.

CULTURE CONDITIONS

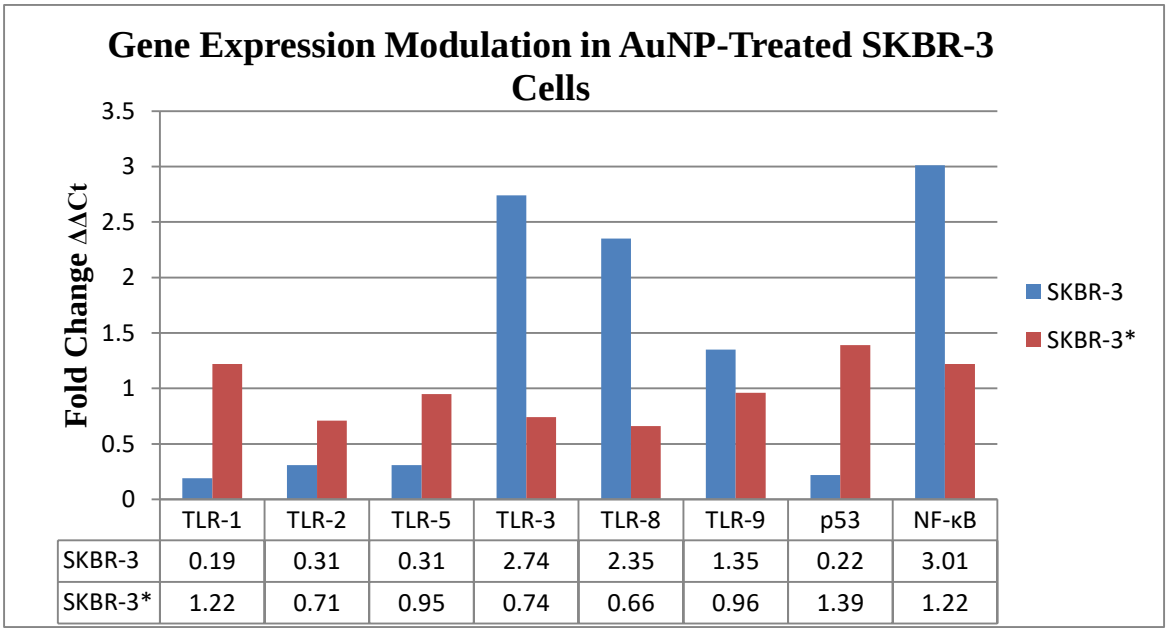
Medium: DMEM + 10% FBS + 2 mM L-glutamine + 100 µg/mL streptomycin + 100 U/mL penicilline. Environment: 37°C, 5% CO_2 , 95% humidity.
TREATMENT: Cells (70% confluency) exposed to 10 µg/mL antibiotic-AuNPs for 24 h. Controls received PBS-only treatment.

GENE EXPRESSION ANALYSIS

Total RNA was extracted using RNeasy Mini Kit (Qiagen) on QIAcube, with DNase I treatment. RNA integrity verified (A260/A280 = 1.9–2.1) While for cDNA Synthesis 1 µg RNA reverse-transcript QuantiTect RT Kit (Qiagen) was used.

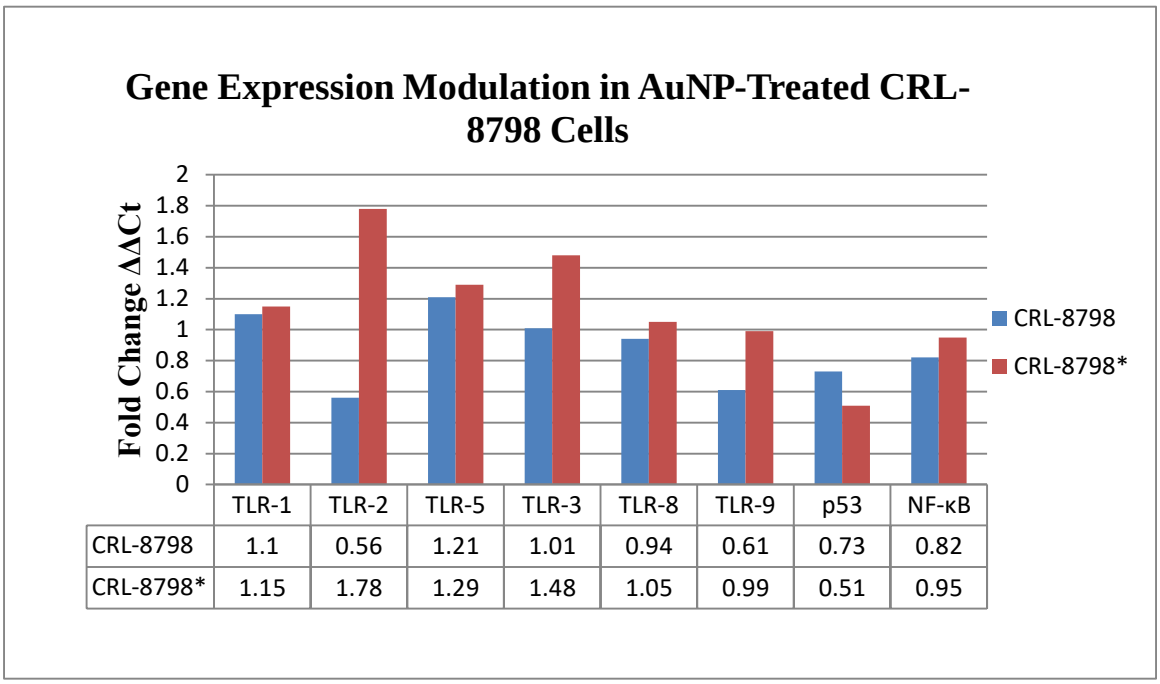
STATISTICAL ANALYSIS
Fold change calculated via 2^{−ΔΔCt} method was employed which demonstrated that Down-regulation values were $0.1 \leq FC \leq 0.5$ while Up-regulation values were $FC \geq 2.0$. Rotor-Gene 6000 Series Software 1.7 was used for expression studies while for Statistical studies Student's "t-test" was performed (SKBR3 vs. CRL-8798); $p < 0.05$ = significant.

RESULTS
CHARACTERIZATION OF ANTIBIOTIC-AUNPS
UV-Vis: Peak absorption at 528 nm confirming spherical AuNPs (diameter ≈ 25 nm).
FTIR: Antibiotic binding evidenced by shifts in amine (1640 cm^{-1} → 1625 cm^{-1}) and carboxyl (1710 cm^{-1} → 1695 cm^{-1}) peaks.
XRD: Bragg peaks at 38.2° (111), 44.4° (200), 64.6° (220), 77.5° (311) confirmed FCC crystalline structure.



	TLR-1	TLR-2	TLR-5	TLR-3	TLR-8	TLR-9	p53	NF-κB
SKBR-3	0.19	0.31	0.31	2.74	2.35	1.35	0.22	3.01
SKBR-3*	1.22	0.71	0.95	0.74	0.66	0.96	1.39	1.22

Figure1: Graphical Representation of the Gene Expression of AuNPs treated SKBR-3 Cells



	TLR-1	TLR-2	TLR-5	TLR-3	TLR-8	TLR-9	p53	NF-κB
CRL-8798	1.1	0.56	1.21	1.01	0.94	0.61	0.73	0.82
CRL-8798*	1.15	1.78	1.29	1.48	1.05	0.99	0.51	0.95

Figure 2: Graphical Representation of the Gene Expression of AuNPs treated CRL-8798 Cells

RESULTS

Gene expression results of qRT-PCR indicate that TLR-1, TLR-2 and TLR-5 and p53 were significantly up-regulated in SKBR3 ($p < 0.01$) while TLR-3, TLR-8 and TLR-9 and NF-κB were uniquely down-regulated, suggesting cancer-specific hyper-activation. TP53 expression increased 3.5-fold in SKBR3, indicating AuNP-induced reactivation of p53-mediated apoptosis. NF-κB was down-regulated (FC = 0.22), suppressing pro-survival pathways. Normal Cells (CRL-8798) exhibited Minimal gene expression changes (all FC 0.8–1.3), confirming AuNP selectivity for cancer cells. AuNPs selectively modulate TLR receptors in CRL-8798 cancer cells align with its role in cancer aggressiveness, potentially serving as a biomarker. Concurrent TP53 up regulation and NF-κB suppression indicate dual pro-apoptotic/anti-inflammatory effects. Antibiotic-AuNPs exhibit cancer-selective gene modulation; down-regulating oncogenic TLR-3, TLR-8 and TLR-9 while up-regulating *TP53*. TLR-1, TLR-2 and TLR-5 up-regulation suggests a compensatory survival mechanism in SKBR3 cells, warranting further study. NF-κB suppression supports AuNP-mediated attenuation of tumor-promoting inflammation.

DISCUSSION

This study demonstrates that antibiotic-conjugated Gold Nanoparticles (AuNPs) selectively modulate innate immune and apoptotic signaling pathways in SKBR3 breast cancer cells, with

minimal effects on normal epithelial cells (CRL-8798). The up regulation of TLR1, TLR2, and TLR5, along with significant TP53 induction, contrasted sharply with the down regulation of TLR3, TLR8, TLR9, and NF-κ B, suggesting a unique cancer-selective gene modulation pattern. These findings provide compelling evidence that AuNPs exert dual anti-cancer effects by promoting apoptosis and suppressing tumor-associated inflammation, reinforcing their potential as precision nanotherapeutics for breast cancer. Toll-like receptors (TLRs) play paradoxical roles in cancer, with some promoting immune surveillance and others contributing to tumor growth, immune evasion, and chronic inflammation (Rakoff-Nahoum & Medzhitov, 2009). The down regulation of TLR3, TLR8, and TLR9 in SKBR3 cells following AuNP treatment is particularly notable. While TLR3 is capable of inducing apoptosis through TRIF-mediated caspase activation (Salaun et al., 2006), it is also known to support cancer cell survival under certain conditions (Chatterjee et al., 2014). TLR8 and TLR9, primarily endosomal receptors, are frequently up regulated in various malignancies, where they stimulate NF-κ B-activation and IL-6/IL-8 exudation and tumor progression (Huang et al., 2015; Basith et al., 2012). In this study, the down regulation of NF-κ B (fold change = 0.22) corroborates the suppression of these pro-inflammatory TLRs. NF-κ B is a key transcription factor that governs cancer cell survival, angiogenesis, and chemo-resistance (Perkins, 2012). Aberrant NF-κ B activation is a hallmark of many solid tumors, including breast cancer (Pratt et al., 2012). The observed suppression of this pathway suggests that AuNPs inhibit cancer-promoting inflammation and immune evasion, consistent with earlier findings where AuNPs blocked NF-κ B nuclear translocation in tumor cells (Zhang et al., 2016). A major finding is the up regulation of TP53 in SKBR3 cells following AuNP exposure. p53 is the "guardian of the genome," functioning as a tumor suppressor that induces apoptosis, cell cycle arrest, and DNA repair in response to cellular stress (Vousden & Prives, 2009). In many cancers, TP53 is mutated or functionally inactivated; hence, its reactivation is a critical therapeutic goal (Levine & Oren, 2009). Notably, normal epithelial cells showed no significant change in TP53 expression, indicating that AuNPs selectively target oncogenic cells while sparing healthy tissue a hallmark of an ideal anti-cancer agent (Dreaden et al., 2012). This selectivity may stem from differences in membrane receptor expression, oxidative stress levels, and endocytosis rates between malignant and non-malignant cells (Conde et al., 2016). Previous studies have shown that functionalized AuNPs can preferentially accumulate in tumor cells due to the enhanced permeability and retention (EPR) effect (Kong et al., 2017), leading to localized modulation of pro-apoptotic pathways. The up regulation of TLR1, TLR2, and TLR5 in SKBR3 cells may reflect a compensatory survival mechanism activated in response to apoptotic stress. These membrane-bound TLRs recognize bacterial-derived ligands such as lipoproteins and flagellin and are often expressed in epithelial and immune cells (Kawasaki & Kawai, 2014).

TLR2 and TLR5 have been implicated in cancer progression through proliferation, cytokine release, and immune modulation (Bhattacharya et al., 2013; Yu et al., 2017). In breast cancer, TLR2 activation has been associated with enhanced cell migration and resistance to therapy (Medzhitov, 2001). While their up regulation in this context may suggest a protective feedback loop, it also raises the possibility that these TLRs could serve as biomarkers of early AuNP-induced stress or as potential co-targets in combinatorial treatments. Further

investigation is needed to determine whether TLR1/2/5 up regulation contributes to residual cell survival or primes the tumor microenvironment for immune recognition. The lack of significant gene expression changes in normal epithelial cells (CRL-8798) (all FC 0.8–1.3) reinforces the tumor-specific action of antibiotic-AuNPs. This specificity is vital for clinical translation, as systemic toxicity remains a barrier for most chemotherapeutics. Moreover, the observed gene expression pattern TP53 upregulation, NF-κB suppression, and selective TLR modulation could serve as a predictive biomarker signature for AuNP sensitivity in HER2+ breast cancers. The ability of AuNPs to down regulate oncogenic TLRs and simultaneously reactivate tumor suppressors aligns with the concept of network-targeted therapy, where multiple cancer hallmarks are addressed in concert (Hanahan & Weinberg, 2011). These findings support the expanding role of nanotechnology in personalized cancer medicine and warrant in vivo validation using xenograft or orthotopic models.

CONCLUSION

This study provides compelling evidence that antibiotic-conjugated Gold-Nanoparticles (Antibiotic-AuNPs) selectively modulate immune-related and tumor-regulatory gene expression in SKBR3 breast cancer cells, while sparing normal cells (CRL-8798), highlighting their cancer-specific therapeutic potential. The significant up-regulation of TP53 and concurrent down-regulation of NF-κB in SKBR3 cells suggests a dual mechanism of action by AuNPs: activation of pro-apoptotic pathways and inhibition of tumor-promoting inflammation. These changes are consistent with the known tumor-suppressive functions of TP53 and the pro-survival roles of NF-κB, indicating effective reprogramming of cellular fate toward apoptosis and immune suppression in cancer cells. The differential modulation of Toll-like receptor (TLR) genes adds an additional layer of insight into the immune-related effects of AuNPs. Specifically, TLR1, TLR2, and TLR5 were significantly up-regulated, potentially reflecting a compensatory or survival response in the cancer cells. In contrast, TLR3, TLR8, and TLR9 were uniquely down-regulated, indicating a suppression of pro-tumorigenic or inflammatory TLR signaling pathways. The selective repression of these TLRs, which have been implicated in tumor aggressiveness and immune evasion, reinforces the notion that AuNPs exert cancer-selective immune-modulatory effects. Notably, normal epithelial cells showed minimal changes in gene expression (fold changes ranging from 0.8–1.3), confirming the biosafety and tumor-specific selectivity of the nano-conjugates. These findings demonstrate that Antibiotic-AuNPs effectively rewire molecular signaling in HER2+ breast cancer cells by simultaneously enhancing tumor suppressor expression and suppressing pro-inflammatory and oncogenic pathways. This dual-action effect offers a promising strategy for targeted breast cancer therapy, especially in tumors characterized by aberrant TLR and NF-κB activity. The study underscores the therapeutic potential of functionalized Nano-materials in precision oncology and highlights the need for further in vivo validation and mechanistic exploration to facilitate clinical translation.

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