ORIGINAL ARTICLE

# COMPARATIVE THERAPEUTIC POTENTIAL OF ANTIBIOTIC LOADED GOLD AND SILVER NANOCONJUGATES IN CANCER THERAPY

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**Background:** Nanomedicine is believed to be an emerging field to improve the efficacy of conventional chemotherapy. Nanoparticles (NPs), particularly drugs conjugated nanometals, have garnered attention as distinct drug carriers in terms of improved pharmacokinetic and pharmacodynamic properties compared to traditional formulations. This study aimed to investigate the potential of silver and gold-loaded antibiotic Azithromycin in demonstrating tumour-suppressing behaviour. **Methods:** Cells after being treated with different working concentrations of drugs/compounds Azithromycin, Au-Azithromycin and Ag-Azithromycin. After obtaining $IC_{50}$ concentrations, treatment groups were further analysed for morphological changes under an inverted phase microscope after 24 and 48 hours of treatment. Finally RT-PCR (Reverse transcription polymerase chain reaction) was performed to evaluate the effect of these treatment groups on the regulation of Rapidly Accelerated Fibrosarcoma-1 (Raf-1), and Mitogen-Activated Protein Kinase 3 (MAPK3) involved in cancer proliferation. GAPDH was used as control gene which is a reference gene used in comparisons of gene expression data. **Results:** Compared to control, all the treatment groups showed very highly significant ($p$<0.001) down regulation of MAPK3 while Raf-1 exhibited highly significant ($p$<0.01) down regulation in all treatment groups. **Conclusion:** Azithromycin loaded with metallic nanoparticles such as gold and silver showed significant suppression of proliferative genes involved in the MAP kinase signalling pathway.

**Keywords:** MAP Kinase pathway, Metallic nano conjugation, Tumour suppression

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## INTRODUCTION

Nanoparticles (NPs) are ultra fine particles (1 to 100 nanometres (nm) in diameter) possessing larger surface area to volume ratio, which render them higher binding efficiencies.[1] In this regard, metal oxide nanoparticles such as gold (Au) and silver (Ag) NPs have been identified to possess suitable diagnostic and therapeutic activities predominantly anticancer and antimicrobial effects, when tested against an extensive range of multidrug-resistant bacteria and cancer cells.[1,2] However, the antibacterial and anticancer doses of these nanoparticles stand low biocompatibility, bioavailability, and biodegradability which eventually limit them to attain an efficient and safe formulation. To optimize these nanoformulations, functionalization of the surface of Ag and Au NPs by biocompatible organic or inorganic materials such as herbs and certain antibiotics has been utilized in recent experiments.[2] On the other hand, cancer cells can bypass antibiotics and chemotherapeutic drugs through several mechanisms which include primarily the overexpression of efflux pumps.

In the past few years, nanotechnology have come up with novel technologies for creating these nanomaterials on the scale of 1–100 nm, which can be employed across different medical domains principally in the treatment of chemotherapy resistant cancer cells.[3] Metallic or metal oxide nanoparticles, especially gold and silver nanoparticles can be reformed through the addition of several therapeutic agents directly or indirectly by photoreactive, hetero-bifunctional, and homo-bifunctional crosslinking reagents.[4] Gold and silver nanoparticles bring about the production of reactive oxygen species (ROS) when given in higher doses which subsequently cause damage to the biological macromolecules such as nucleic acids, proteins, and enzymes while in low doses it results in activation of cell signalling with low cytotoxicity.[5]

Chemotherapy shows an essential role in the management of cancer, but the development of resistance against chemotherapeutic agents, the adverse effects, and non-specific toxicity impend the effectiveness of anticancer agents. Hence, it is pivotal to develop novel formulations or repurpose already marketed conventional drugs with known adversities for effective chemotherapeutics.

Consequently, the use of multifunctional agents, comprising antimicrobials, biocompatible materials, and gold or silver nanoparticles, may result in a decrease in the adverse effects of chemotherapy. This is attributed to accomplishing definite tumour-targeting and reverting drug resistance.[6] Subsequently, antibiotic conjugated nanoparticle could be convincing bactericidal and tumouricidal materials.[7]

One study documented the comparative cytotoxic effect of Cefotaxime conjugated Ag-Nps on human RPE-1 normal cells and human MCF-7 breast cancer cell lines which surprisingly exhibited pronounced cytotoxic activity on cancer cells while staying safe to normal cells.[8] Macrolides beyond their classical antibacterial activity, have been reported to demonstrate potential against both drug-sensitive and drug-resistant cancers via modulating diverse targets and signalling pathways.[9] Particularly, Azithromycin has distinct properties as compared to other macrolides such as slow release in cells, higher local concentration, better safety profile, and longer half-life which make it an ideal to be repurposed as chemotherapeutic agent.[7]

Keeping in view this background, in the present study we investigated the activities of Azithromycin with advanced targeted drug delivery such as gold and silver nanoconjugated forms of Azithromycin on morphology and proliferative pathway HepG2 cancer cells.

## METHODOLOGY

This was an *in vitro* experimental study conducted at MDRL, Ziauddin University, Karachi for 8 months from July 2019 to February 2020 after institutional approval from ERC, Ziauddin University (Reference code: 0750119DZPHA).

Human hepatocellular cell line (HepG2) was generously provided by Biobank facility of Dr. Panjwani Centre for Molecular Medicine and Drug Research (PCMD), ICCBS, University of Karachi. All chemicals were purchased from Thermo Scientific, USA. Imaging was done under Floid cell imaging system, Thermo Scientific, USA.

Synthesis of Azithromycin conjugated gold and silver nanoparticles were synthesized at the Hussain Ebrahim Jamal (HEJ) Research Institute of Chemistry, University of Karachi by $NaBH_4$ reduction method. Azithromycin conjugated gold and silver nanoparticles were characterized by UV-visible spectroscopy, Fourier Transform Infrared Analysis (FTIR), Atomic Force Microscopy (AFM), and Dynamic Light Scattering (DLS) analysis.

Stock concentrations of Azithromycin were prepared in sterile 100% DMSO while stock concentrations of silver and gold conjugated forms of Azithromycin were made in ethanol. Cells after being treated with different working concentrations of Azithromycin and its conjugated forms yielded final $IC_{50}$ concentrations which came out to be 88.8 µg/ml, 78.3 µg/ml, and 33.3 µg/ml for Azithromycin, Au-Azithromycin and Ag-Azithromycin respectively.

Control and treatment groups were observed for morphological changes under inverted phase microscope after 24 and 48 hours of treatment. RT-PCR was performed to evaluate comparative suppressive effect of these treatment groups on tumour oncogenes.

RT-PCR was performed to evaluate the impact of the tested drugs on the underlying proliferative pathway of cancer progression, i.e., MAPK pathway. RNA was isolated from the control and treated HepG2 cells by the TRIzol method followed by cDNA synthesis and real time PCR as per manufacturer's instructions. Each sample was run in triplicate and GAPDH was used as the normalizing control gene. Primers were designed via Primer3 software. Primers for Raf-1, MAPK3 and GAPDH are listed in Table-1. The real time PCR data was analysed using the $2^{-\Delta\Delta CT}$ relative quantification method to calculate the fold change in gene expression.[10]

Statistical software SPSS version 20.0 was used for analysis of data. All numerical values were presented as Mean±SEM which were generated by ANOVA. In order to find comparison between the groups, Tukey's post hoc test was applied, and $p \leq 0.05$ was considered significant.

**Table-1: Primers for RAF-1, MAPK3 and GAPDH**

| Gene | Gene name | Primer sequence |
|---|---|---|
| **GAPDH** | Glyceraldehyde-3 Phosphate Dehydrogenase | (Forward) CCAGAACATCATCCCTGCCT (Reverse) CCTGCTTCACCACCTTCTTG |
| **MAPK3** | Mitogen-Activated Protein Kinase 3 | (Forward) GGCCCGAAACTACCTACAGT (Reverse) CGTCGGGTCATAGTACTGCT |
| **Raf-1** | Raf-1 Proto-Oncogene, Serine/Threonine Kinase | (Forward) CAACCCCAGAGCAATTCCAG (Reverse) AGGTGTTTGTAGAGGCTGCT |

## RESULTS

The effect of Azithromycin, Au-Azithromycin and Ag-Azithromycin cellular morphology was observed under inverted phase microscope after 24 and 48 hours of treatment. The control cells displayed normal morphology of HepG2 cells. The cells treated with Au-Azithromycin and Ag-Azithromycin (at their $IC_{50}$ concentrations) showed morphological alteration like cellular swelling and change of shape from elliptical to round compared to control.

RT-PCR was performed to determine the effects of $IC_{50}$ of Azithromycin, Au-Azithromycin and Ag-Azithromycin on the expression of oncogenes Raf-1 and MAPK3 related to MAPKinase pathway involved in hepatic cancer proliferation. As compared to control, all the treatment groups showed very highly significant ($p<0.001$) down regulation of MAPK3 while Raf-1 exhibited highly significant ($p<0.01$) down regulation in all treatment groups.

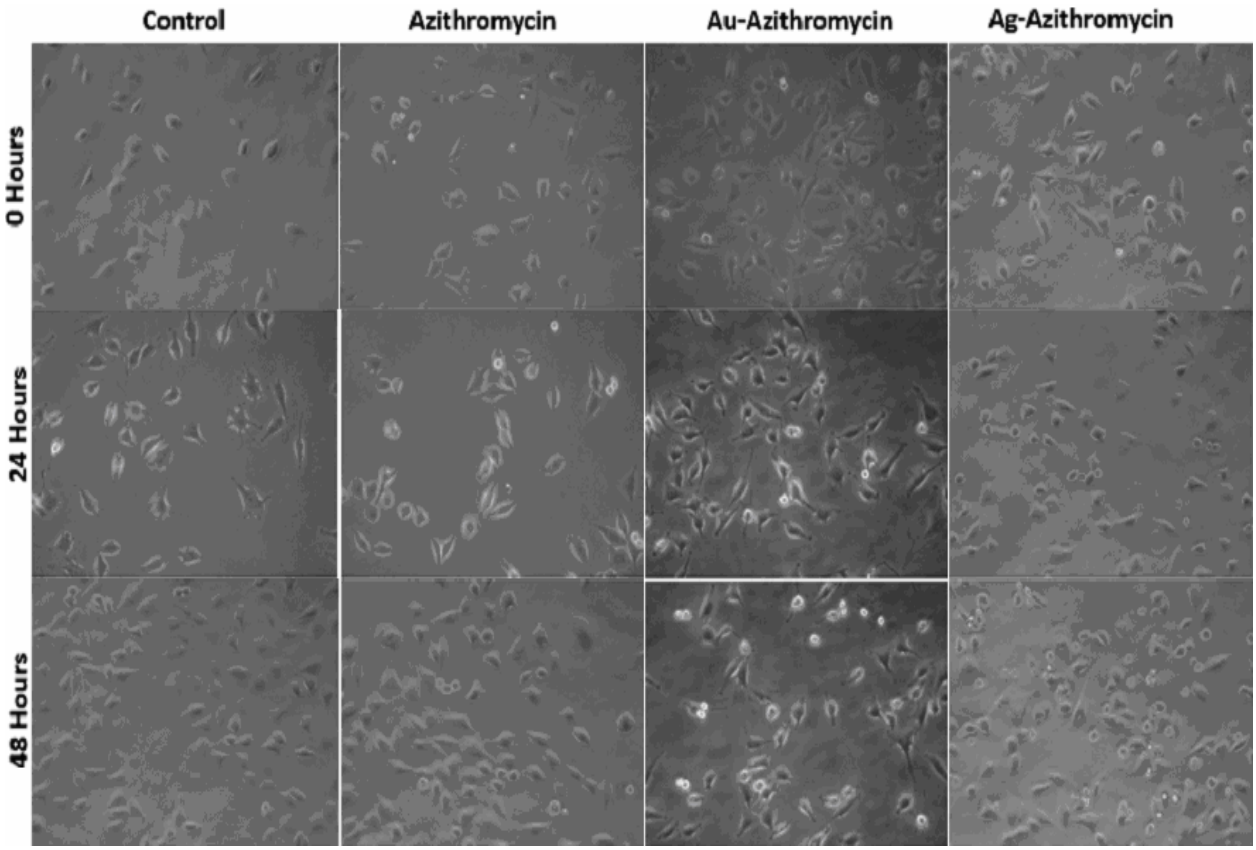


**Figure-1: Morphological changes in HepG2 cells after treatment with Azithromycin, Au-Azithromycin and Ag-Azithromycin at 0, 24, and 48 hours.** (Images obtained at 10× magnification under inverted phase microscope)

**Table-2: ΔΔCT and fold change values for AZM treated group**

| | | ΔCT=GOI-HKG | Average | ΔΔCT=Treated-Untreated | Fold change | Average |
|---|---|---|---|---|---|---|
| **Raf-1** | **Control** | 12.13 | 12.35 | | 1 | |
| | **Control** | 12.53 | | | 1 | |
| | **Control** | 12.40 | | | 1 | |
| | **AZM** | 16.25 | | 3.896667 | 0.067141 | 0.162982 |
| | **AZM** | 14.02 | | 1.666667 | 0.31498 | |
| | **AZM** | 15.58 | | 3.226667 | 0.106826 | |
| **MAPK3** | **Control** | 10.73 | 10.32 | | 1 | |
| | **Control** | 10.26 | | | 1 | |
| | **Control** | 9.99 | | | 1 | |
| | **AZM** | 16.32 | | 5.99 | 0.486327 | 0.033559 |
| | **AZM** | 14.33 | | 4.01 | 0.151774 | |
| | **AZM** | 15.77 | | 5.45 | 0.432269 | |

**Table-3: ΔΔCT and fold change values for Au-AZM treated group**

| | | ΔCT=GOI-HKG | Average | ΔΔCT=Treated-Untreated | Fold change | Average |
|---|---|---|---|---|---|---|
| **Raf-1** | **Control** | 12.13 | 12.35 | | 1 | |
| | **Control** | 12.53 | | | 1 | |
| | **Control** | 12.40 | | | 1 | |
| | **Au-AZM** | 10.32 | | -2.03333 | 0.24429 | 0.27463 |
| | **Au-AZM** | 10.63 | | -1.72333 | 0.302848 | |
| | **Au-AZM** | 10.50 | | -1.85333 | 0.276752 | |
| **MAPK3** | **Control** | 10.73 | 10.32 | | 1 | |
| | **Control** | 10.26 | | | 1 | |
| | **Control** | 9.99 | | | 1 | |
| | **Au-AZM** | 10.73 | | 0.403333 | 0.756109 | 0.67284 |
| | **Au-AZM** | 11.21 | | 0.883333 | 0.542113 | |
| | **Au-AZM** | 10.80 | | 0.473333 | 0.720298 | |

**Table-4: ΔΔCT and fold change values for Ag-AZM treated group**

| | | ΔCT=GOI-HKG | Average | ΔΔCT=Treated-Untreated | Fold change | Average |
|---|---|---|---|---|---|---|
| **Raf-1** | **Control** | 12.13 | 12.35 | | 1 | |
| | **Control** | 12.53 | | | 1 | |
| | **Control** | 12.40 | | | 1 | |
| | **Ag-AZM** | 12.40 | | -0.2 | 0.870551 | 0.734253 |
| | **Ag-AZM** | 13.00 | | 0.4 | 0.757858 | |
| | **Ag-AZM** | 13.40 | | 0.8 | 0.574349 | |
| **MAPK3** | **Control** | 10.73 | 10.32 | | 1 | |
| | **Control** | 10.26 | | | 1 | |
| | **Control** | 9.99 | | | 1 | |
| | **Ag-AZM** | 10.00 | | -0.32667 | 0.797377 | 0.60616 |
| | **Ag-AZM** | 11.20 | | 0.873333 | 0.545884 | |
| | **Ag-AZM** | 11.40 | | 1.073333 | 0.47522 | |

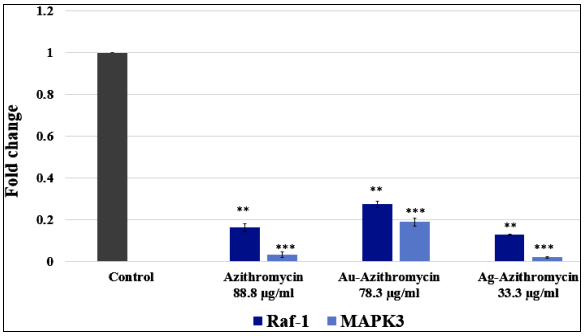


**Figure-2: Suppressive effect of Azithromycin, Au-Azithromycin and Ag-Azithromycin on mRNA expression of Raf-1 and MAPK3 oncogenes**

## DISCUSSION

Conventional antibiotics, especially macrolides are reported to modulate immunity and alter oxidative balance in the body apart from bactericidal activities[11], consequently may play a role in managing chronic obstinate conditions such as cancers[12]. On the same side, advancements in nanotechnology have transformed the scope of cancer treatment since they ensure drug biocompatibility, safety, stability, and improved permeation. By utilizing both approaches, one can better handle this deleterious disease. Usman *et al*[13] revealed the cytotoxic activity of Azithromycin, Au-Azithromycin, and Ag-Azithromycin against HepG2 cancer cells at $IC_{50}$ concentrations of 88.3 µg/dL, 78.8 µg/dL and 33.3 µg/dL respectively. The current study was designed to appraise the suppressive potential of these drugs against oncogenes (Raf-1 and MAPK3) belonging to MAP-Kinase pathway involved in the proliferation of hepatic cancer cells. Multiple studies supported the anticancer efficacy of Azithromycin, and demonstrated its antiproliferative, pro-apoptotic, anti-autophagy, and anti-angiogenic effects in various cancer cell lines such as colon cancer cell lines (HCT-116, SW480), cervical cancer cell line (Hela) and gastric cancer cell line (SGC-7901).[14,15] When used in combination, it was also found to potentiate the anticancer effects of other chemotherapeutic agents.[14,16] Previous literature also reported the potential role of other macrolides *viz* clarithromycin (CAM) and erythromycin on human hepatoma cells (HepG2) and chemically induced hepatocellular carcinoma in rats as well.[17,18]

The results of the current study depicted the very highly significant down-regulation of MAPK3 (a proliferative proto-oncogene) and, highly significant down-regulation of Raf-1 by all treatment groups as compared to control (Figure-2).

Usma *et al*[13] also explored the additive effects of combining Azithromycin with the chemotherapeutic agent Sorafenib with optimistic findings. The reason for appraising the anticancer effects of azithromycin-loaded gold and silver conjugated forms apart from Azithromycin alone is that combining the nano-drug carrier, or magnetic characteristics of NPs with conventional drugs produces a hybrid product that shows enhanced and precise drug delivery.[19] Delivery of anticancer agents exactly to tumours poses a significant challenge due to the tendency of most drugs to cause off-target effects, leading to non-specific cell death. Multi-functionalized metallic NPs have been explored as a new carrier system in the era of cancer therapeutics.[20] Gold and silver particles have shown great capabilities in both cancer diagnostics and therapeutics. Au-NPs alone have beset tumour cells in brain, breast, and colon cancer cell lines via inhibition of different cancer proliferative pathways such as PI3K/Akt, MAP Kinase signalling, and VEGF pathway.[21] Ag-NPs imparted cytotoxic effects in human hepatoma cells, lung cancer, breast cancer, and cervical carcinoma by decreasing mitochondrial function, ROS production, cell cycle deregulation, and induction of apoptotic genes.[22,23] The combination of nanoparticles with antimicrobials particularly Azithromycin is anticipated to hold promise as anti-cancer agent potentially demonstrating efficacy against tumour cells enabling more precise targeted delivery.

## CONCLUSION

Azithromycin loaded gold and silver particles emerged as potent antiproliferative agent against the HepG2 cell line via inhibition of genes related to MAPK pathway. Further studies are warranted to comprehensively understand and validate these findings for further therapeutic implications.

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### Contribution of Authors:

**DZ:** Performed cell culture experiments, literature search
**SU:** Research idea and study design, supervised experiments, mentorship
**KI:** Final drafting and revision
**SK:** Data interpretation
**SHZ:** Statistical analysis/interpretation
**SA:** Data acquisition
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