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# SYNTHESIS, CHARACTERISATION AND INVESTIGATION OF THE ANTICANCER POTENTIAL OF CARMOFUR-LOADED SILVER NANOPARTICLES

## KARMOFUR YÜKLÜ GÜMÜŞ NANOPARTİKÜLLERİNİN SENTEZİ, KARAKTERİZASYONU VE ANTİKANSER POTANSİYELİNİN ARAŞTIRILMASI

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### ABSTRACT

**Objective:** The term "triple-negative breast cancer" (TNBC) is used to describe tumours that do not express oestrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2). TNBC tends to be more aggressive than other types of breast cancer. Current antineoplastic drugs have limited treatment options for malignant breast cancer owing to their narrow therapeutic index, toxicity, resistance, and non-selectivity. Therefore, there is a need for the prompt development of new medicinal drugs for TNBC. Here, we investigated the growth inhibition potential of carmofur-bonded silver nanoparticles (AgNPs-Car) on two TNBC cell lines, MDA-MB-231 and 4T1, and compared the effects with non-cancerous Human umbilical vein endothelial cells (HUVECs).

**Material and Methods:** AgNPs-Car were synthesised and characterised by FTIR, DLS, SEM, and EDX. The anticancer effect was evaluated using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.

**Results:** AgNPs-Car was determined to be predominantly more effective than Car alone. Mainly, 4T1 cells were 5.7-fold more sensitive to AgNPs-Car than Car alone. While AgNPs showed no considerable toxicity on HUVECs, they significantly induced the cytotoxicity of MDA-MB-231 and 4T1 cells.

**Conclusion:** Our results showed that AgNPs-Car is a promising anticancer agent due to its highly potent and selective growth inhibitory effect on TNBC cells.

### ÖZET

**Amaç:** "Üçlü negatif meme kanseri" (TNBC) terimi, östrojen reseptörü (ER), progesteron reseptörü (PR) veya insan epidermal büyüme faktörü reseptörü 2'yi (HER2) eksprese etmeyen tümörleri tanımlamak için kullanılır. TNBC, diğer meme kanseri türlerinden daha agresif olma eğilimindedir. Malign meme kanserinde güncel antineoplastik ilaçların tedavi indeksinin kısıtlayıcı olması, toksisitesi, direnci ve seçici olmaması nedeniyle sınırlı tedavi seçenekleri bulunmaktadır. Bu nedenle, TNBC için yeni tıbbi ilaçların derhal geliştirilmesine ihtiyaç vardır. Burada, MDA-MB-231 ve 4T1 olmak üzere iki TNBC hücre hattı üzerinde karmofur bağlı gümüş nanopartiküllerin (AgNP-Car) büyüme inhibisyonuna etkisini araştırdık ve etkinliklerini kanserli olmayan insan göbek damarı endotel hücreleri (HUVEC) ile karşılaştırdık.

**Gereç ve Yöntem:** AgNP-Car, FTIR, DLS, SEM ve EDX ile sentezlendi ve karakterize edildi. Anti-kanser etkisi 3-(4,5-Dimetiltiyazol-2-il)-2,5-Difeniltetrazolium Bromür (MTT) testi ile değerlendirildi.

**Bulgular:** AgNP-Car'ın nanopartiküllerin tek başına karmofurdan ağırlıklı olarak daha etkili olduğu belirlendi. Özellikle AgNP-Car uygulanmış 4T1 hücrelerinin, karmofur uygulanmış gruplara göre 5,7 kat daha duyarlı olduğu belirlendi. AgNP'ler HUVEC'ler üzerinde önemli bir toksisite göstermezken; MDA-MB-231 ve 4T1 hücrelerinin sitotoksisitesini önemli ölçüde indüklediler.

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**Sonuç:** Sonuçlarımız, AgNP-Car'ın TNBC hücreleri için oldukça güçlü ve seçici büyüme inhibitörü etkisi nedeniyle umut verici bir anti-kanser ajanı olabileceğini göstermektedir.

**Anahtar kelimeler:** Gümüş nanopartiküller, antiproliferatif, antikanser, karmofur, üçlü negatif meme kanseri hücreleri

## INTRODUCTION

Among all cancer types, breast cancer is the most common type among women, and it is very likely to cause death when it progresses (1). Therefore, breast cancer remains one of the most critical health problems in many countries. Triple-negative breast cancer (TNBC) is the most malignant and mortal type of breast cancer compared to its subtypes. TNBCs are known for the deficiency of the expression of oestrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2). TNBC exhibits significant genetic heterogeneity. Mutations in key oncogenes and tumour suppressor genes such as *TP53*, *BRCA1/2*, and *PIK3CA* contribute to treatment resistance. Epigenetic changes, including DNA methylation and histone modifications, also play a role in silencing genes critical for drug response. Abnormal activation of signaling pathways such as PI3K/AKT/mTOR, JAK/STAT, and NF-κB promotes the survival and proliferation of TNBC cells. These pathways often confer resistance by enabling cells to evade apoptosis and sustain growth despite treatment (2-4). TNBC is also defined as aggressive with the rate of upper metastasis and poor prognosis. Overexpression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp) and ABCG2, enables TNBC cells to actively pump out chemotherapeutic agents, reducing intracellular drug concentrations (3). The treatment options for TNBC are minimal due to the absence of molecular targets; therefore, these patients cannot be treated with endocrine therapy targeting HER2, and TNBCs are treatment-resistant cancers to usual therapies like chemotherapy and immunotherapy. Consequently, there is a need to develop new treatment approaches for such types of aggressive breast cancer.

The development of nano drug delivery systems can provide great opportunities through the ability of targeted drug delivery and tumour responses to overcome the clinical challenges observed, such as drug resistance of TNBCs (5). By encapsulating drugs within nanoparticles (NPs), nanocarriers can bypass efflux pumps and increase intracellular drug accumulation. For example, nanoparticle formulations of doxorubicin have been shown to evade P-gp-mediated efflux in resistant TNBC cells (3). NPs may selectively affect the tumour tissue because of enhanced permeability and retention (EPR). Hence, the side effects of chemotherapeutics might be decreased (6). High-dose drugs reach cancer cells to improve therapeutic outcomes while reducing systemic toxicity through delivery systems with lower drug concentrations (5). By selectively targeting TNBC cells and sparing normal tissues, nanodrug delivery systems minimise off-target effects and reduce the toxicity associated with conventional chemotherapy, thus improving patient quality of life.

A drug that has been investigated in treating breast cancer in recent years is carmofur (Car). Car (1-hexylcarbamoyl-5-fluorouracil), a masked form of 5-fluorouracil (5FU), is a pyrimidine analog known as an antineoplastic agent used to treat several cancers (7). Generally, the lipophilic form of 5FU can be taken orally (8). In some countries, Car has also been used as an adjuvant chemotherapy for breast and colorectal cancer (9, 10). In addition, it is a highly strong acid ceramidase (AC) inhibitor (11). The enzyme ceramidase is crucial in sphingolipid synthesis and catalyses the ceramide into sphingosine and fatty acids. Ceramide is a messenger that activates apoptosis and cell differentiation (11-13). Furthermore, it has been reported that Car is more effective in targeting 5FU-resistant cells (14). A hexyl carbamoyl substituent serves as a transport form in the structure of this antimetabolite/Car and supports the entry of 5FU into cells (15). Due to its properties, carmofur has long been used to treat human cancer. In addition, the potential of Car to inhibit cancer cell proliferation has been recognised as an independent attribute of its ability to produce 5FU (16). These factors make it an essential tool for studies in cancer research, supporting the notion that ceramidase should be inhibited for medicinal purposes. Besides all these, AgNPs synthesised with Car have not been evaluated in TNBC or cancer treatment.

Nanocarriers may increase the drug half-life in the biological environment, display enhanced pharmacokinetic activity, and exhibit better patient compliance (17). Besides, nanodrugs prepared with Car or other drugs differ due to some advantages of their physicochemical structure, and they cause various cellular responses in different cell types (18). Among the many nanoparticles, silver nanoparticles have recently attracted significant attention due to their uniqueness and their multiple applications in other fields such as biomedical, food, cosmetics, and engineering (19, 20).

The synthesised silver nanoparticles (AgNPs) altered in size, shape, and other physiological characteristics. The nanosized particles can be catalytic and have electromagnetic capability

many more times. It is considered that the alteration in size plays a significant role in the nanoparticle activity. In addition, the nanoparticle concentration range and agglomeration are important components affecting toxicity induction (21-24). On the other hand, AgNPs have been shown to cause cytotoxicity in a dose-dependent manner (25).

The development of resistance remains a significant challenge for treating TNBC. However, advances in nanotechnology-based drug delivery systems offer innovative solutions to address these hurdles. By enabling targeted delivery, overcoming resistance mechanisms, and improving drug efficacy, nanomedicine holds great potential to transform the therapeutic landscape for TNBC and improve patient outcomes. Therefore, we used AgNPs as drug carriers because of their convenient and low-cost synthesis and biocatalytic-photocatalytic advantages in this study (26-28). We used the highly aggressive MDA-MB-231 (human) and 4T1 (murine) cells as the TNBC cell model (29, 30). We synthesised carmofur-bonded silver nanoparticles (AgNPs-Car), a nanoparticle that has never been found in the literature, with AgNP and Car. Then, we studied the anticancer effects of AgNPs-Car on highly malignant and drug-resistant MDA-MB-231 human breast tumour cells, 4T1 mouse breast tumour cells, and normal HUVEC cells as control cells to investigate whether there is a new treatment option in TNBCs.

## MATERIALS AND METHODS

The MDA-MB-231, 4T1, and HUVEC were purchased from American Type Culture Collection (ATCC, USA). Sodium borohydride ($NaBH_4$), silver nitrate ($Ag(NO_3)$), sodium hydroxyl (NaOH), and trisodium citrate (TSC) were obtained from Sigma Aldrich (USA), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) kit, dimethylsulphoxide (DMSO), and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma (USA). Trypsin, streptomycin (S), penicillin (P), foetal bovine serum (FBS), and phosphate buffer saline (PBS) were obtained from Gibco and Car from Glentham (UK).

### Synthesis of the AgNPs-Car

$1x10^{-3}$ M $Ag(NO_3)$, $4.3x10^{-3}$ M trisodium citrate, and $2x10^{-3}$ M $NaBH_4$ were prepared and used as stock solutions. The synthesis of AgNPs and AgNPs-Car was prepared using various concentrations of $NaBH_4$, TSC, and $Ag(NO_3)$. In a dark room, they were combined for 30 min. at 60°C. The $Ag(NO_3)$ solution was then added at 90°C drip-by-drip. A 0.1 M NaOH solution was used to bring the pH of the solution down to 10.5 before it was centrifuged at 12.000 rpm for 15 min., rinsed with deionised water, and stored at +4°C.

### Characterisation of AgNPs-Car

The Fourier transform infrared spectroscopy (FTIR) spectra of the AgNPs and AgNPs-Car were analysed by a Perkin Elmer Spectrum 400 spectrometer with 4 $cm^{-1}$ resolution and ten scans per spectrum. FTIR analyses were taken later, and the samples were dried.

Dynamic light scattering (DLS) examined the nanoparticle size and size distribution of the samples and zeta sizer showed the evaluations at 25°C (Malvern Instruments Ltd., Malvern, UK). The samples were dripped onto the silicon base material for the scanning electron microscope (SEM) analysis. The samples were left overnight to enable the drying of the liquid. Later, the samples were coated with Au/Pd to increase their surface conductivity. SEM and Energy dispersive X-ray (EDX) analysis was started after the samples were prepared with this procedure.

### Cell culture analysis

MDA-MB-231, 4T1, and HUVEC cells were used in this study. The cell lines were seeded in T75 flasks with DMEM, including 10% FBS and 1% P/S in 5% $CO_2$ and 95% relative humidity incubator at 37°C. The cells were treated when they reached 80% confluency.

Cell viability differences in Car, AgNPs, and AgNPs-Car were determined by MTT after 72 h. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide reduced by live cells to formazan in the MTT, a colorimetric test (31, 32). The subsequent concentrations of Car (3.12, 6.25, 12.5, 25, 50, 70 µg/mL) and AgNPs-Car (3.12, 6.25, 12.5, 25, 50 µg/mL) were applied on MDA-MB-231, 4T1, and HUVEC (Table1). The Ag concentration in AgNPs-Car is shown in Table 1. MTT solution (10 µl of 5 mg/mL PBS) was added, and the cells were incubated at 37°C for 4 h; the medium was taken, and 100 µL DMSO, which is used to dissolve formazan crystals, was added for lysing. The absorbance was read at 570 nm. The trials were done thrice. Dose-response curves were created to determine the $IC_{50}$ (concentration that inhibits the growth of 50% of cells) for AgNPs-Car. This metric was used to evaluate the effectiveness of AgNPs-Car. The $IC_{50}$ was calculated using the $IC_{50}$ Calculator (AAT Bioquest) (https://www.aatbio.com/tools/ic50-calculator). A Nikon Eclipse Ts2 microscope evaluated the morphological changes.

**Table 1:** AgNPs-Car concentrations used in the cytotoxicity tests

| Car stock | AgNPs stock | AgNPs-Car stock | AgNPs-Car $^1/_2$ diluted | AgNPs-Car $^1/_4$ diluted | AgNPs-Car $^1/_8$ diluted |
|---|---|---|---|---|---|
| 1 mg/mL | 108 µg/mL | 50 µg/mL Car+ 5.4 µg/mL Ag | 25 µg/mL Car+ 2.7 µg/mL Ag | 12.5 µg/mL Car + 1.3 µg/mL Ag | 6.25 µg/mL Car +0.6 µg/mL Ag |

Car: Carmofur, AgNPs: Silver nanoparticles, AgNPs-Car: Carmofur loaded silver nanoparticles, Ag: silver

### Statistical analysis

One-way ANOVA performed for multiple comparisons of the results of the experiments using the GraphPad Prism 9.1.0 program. The mean of the control group was compared to the mean of the treated groups using the Student's t-test. A $p<0.05$ was considered statistically significant. Results are given as mean ± S.D. At least three runs of each test were performed.

## RESULTS

### The AgNPs displayed proper characterisation results for cancer cell implementation

FTIR results demonstrate the data of AgNPs (Figure 1a) and AgNPs-Car (Figure 1b). In Figure 1a, the first peak was illustrated at 3251 $cm^{-1}$ for the -OH band, the second peak was measured at 1636 $cm^{-1}$ for the carboxylate group of sodium citrate, and the band for -CH vibrations at lower wavelengths. Shifts were detected in Figure 1b compared with Figure 1a. The -OH vibration band determined the amine groups in the AgNPs-Car structure by which shifted from 3251 to 3253 $cm^{-1}$. Furthermore, characteristic vibration peaks, such as -CN stretching, -OH bending, -CH bending, and -CO stretching, were measured between 1637-1015 $cm^{-1}$ in the AgNPs-Car group.

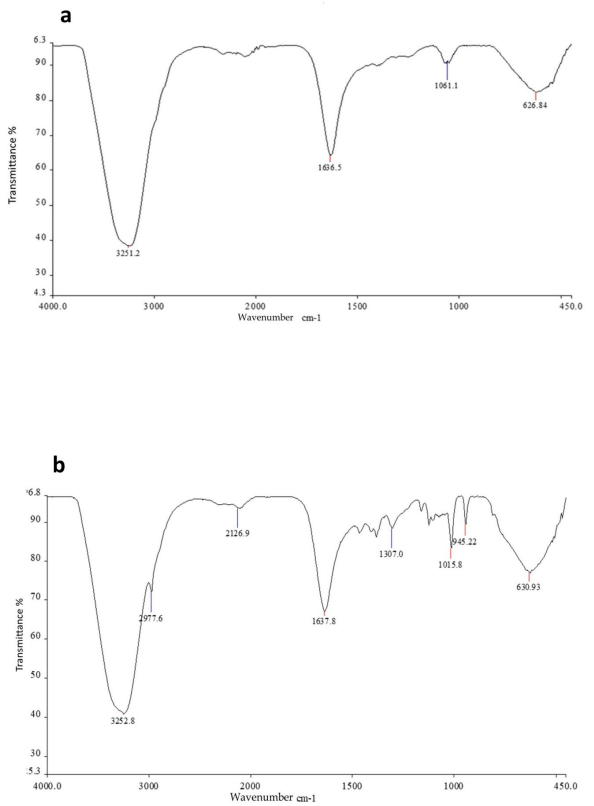


**Figure 1:** Vibration peaks were determined by FTIR spectroscopic analysis in **(a)** AgNPs **(b)** AgNPs-Car groups

At the same time, while the characteristic vibration signal peak of the AgNPs was seen at 626 $cm^{-1}$ in Figure 1b, it was also determined that this vibration signal shifted to 630 $cm^{-1}$ after drug loading.

### Car decreased the AgNPs size ideally

Figure 2a shows, however, that when Car was added to the AgNPs in Figure 2b, the particles were found to be more dispersed. Its dimensions were found to range from 10 to 20 nm. This confirms our zeta sizer measurements (Figure 3a, b). The nanoparticle size was examined after being diluted with distilled water. The light scatter measurements were evaluated at 25°C. A Nano ZS and DLS determined the size and size distribution of the AgNPs in the samples. In this study, the average AgNPs-Car size was around 10 nm. As a result of DLS analysis, AgNP and AgNP-Car showed a homogenous distribution in length (Figure 3).

The EDX analysis in Figure 4a shows, together with the base material silicon, that the amount of silver attached to the surface of this base material is around 2%. In Figure 4b, while the amount of silver decreases to 0.5%, the

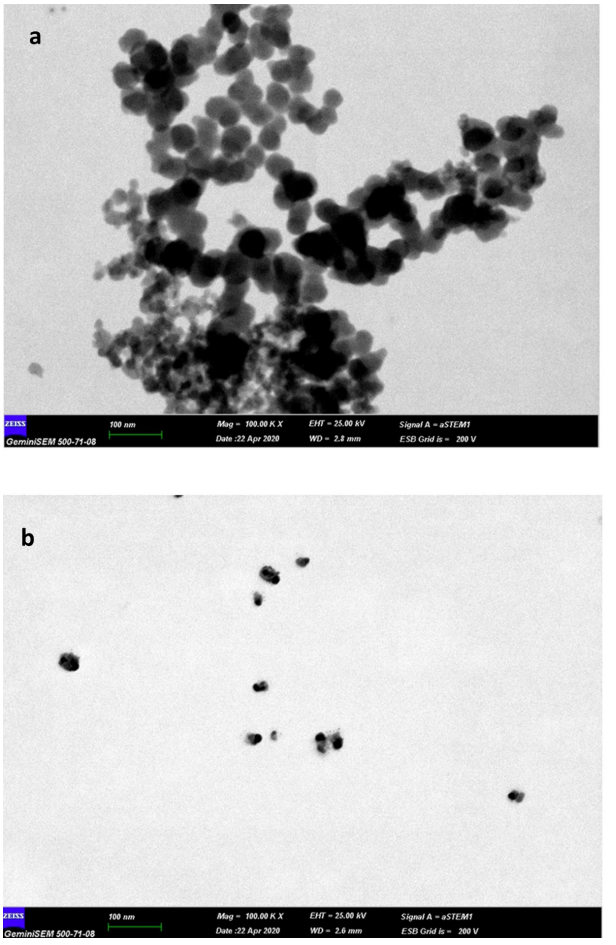


**Figure 2:** SEM images of **(a)** AgNPs and **(b)** AgNPs-Car

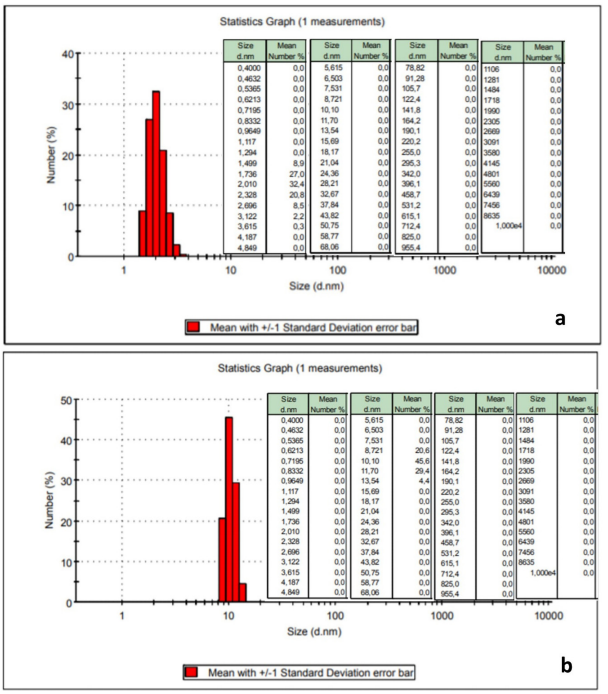


**Figure 3:** The DLS analyses of **(a)** AgNPs and **(b)** AgNPs-Car

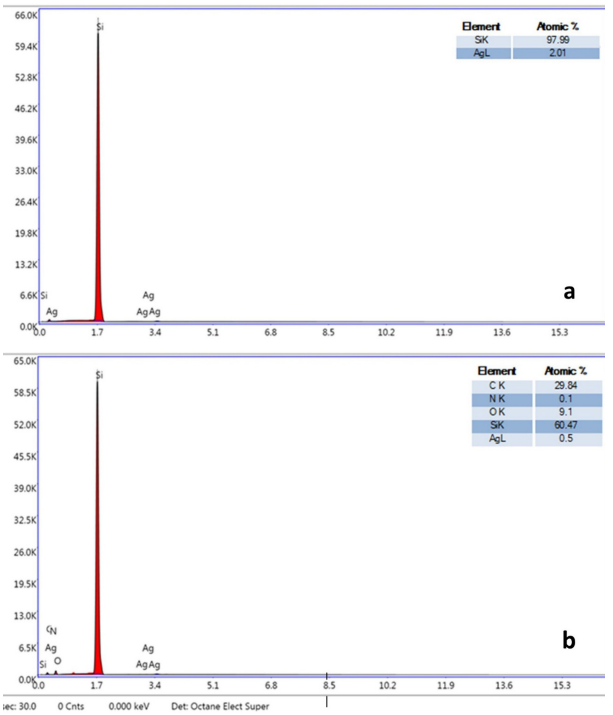


**Figure 4:** EDX analysis of **(a)** AgNPs and **(b)** AgNPs-Car

amount of the elements belonging to the AgNPs-bound Car in percentage are also seen on the surface. Elements such as carbon, nitrogen, and oxygen are known to be included in the structure of Car, which confirmed that we successfully bound our drug to AgNPs.

**AgNPs-Car are effective agents for cytotoxicity on chemotherapy-resistant TNBC cells**

Figure 5 shows the antiproliferative effects of separately Car, AgNPs, and AgNPs-Car that synthesised against MDA-MB-231 and 4T1 cells. As shown in Table 2, AgNPs-Car is several times more effective than Car alone on treatment-resistant TNBC cells. In addition, the fact that AgNPs (without Car) cause no considerable cytotoxicity in any cell group (Figures 5 and 6) indicates that AgNPs will not cause side effects in normal body cells.

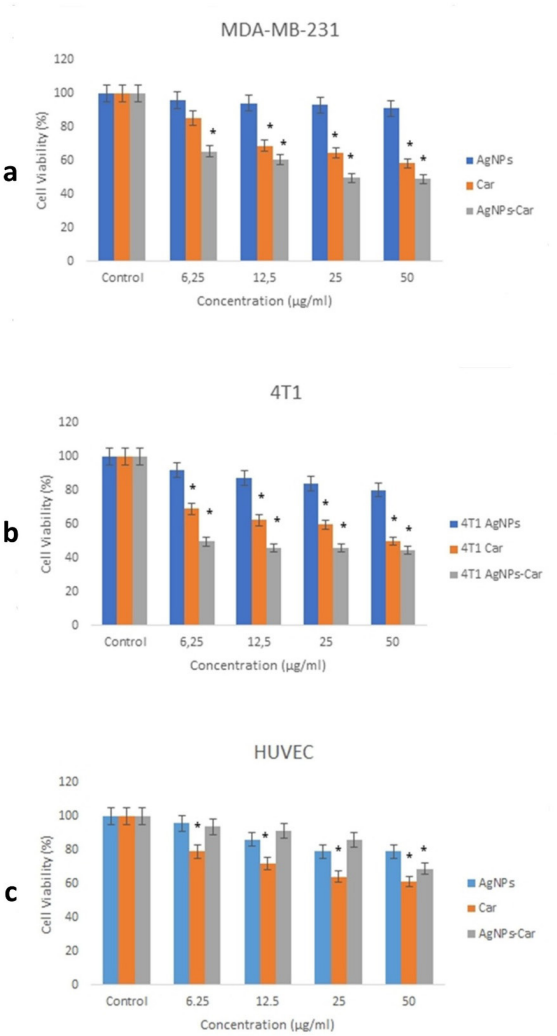


**Figure 5:** Viability (%) of MDA-MB-231 **(a)**, 4T1 **(b)** cancer cells, and HUVEC healthy cells **(c)** incubated with AgNPs, Car, and AgNPs-Car at various concentrations for 72 h. The Car concentrations are indicated on the abscissa of the Figure. AgNPs are also added to the Figure for comparison of the cytotoxicity of the agents, although there is no Car. P<0.05 (*) demonstrated statistical significance compared to the control group. The samples were examined using the Student's t-test. GraphPad Prism 9.1.0 was used for the statistical analysis (GraphPad Software). At least three runs of each test were performed

Notably, AgNPs showed almost no toxicity to non-cancerous HUVEC cells (Figure 6). As mentioned before, many factors determine the toxicity of AgNPs. In addition, the dose of Ag in AgNPs-Car that we used at $IC_{50}$ concentration is relatively low (3.1 µg / mL for MDA-MB-231, 0.8 µg / mL for 4T1) (Figure 7).

**Healthy cells were not affected by AgNPs-Car; therefore, they are ideal agents for breast cancer cell therapy**

In our results, Car appears to have an anticancer effect on treatment-resistant MDA-MB-231 and 4T1 breast cancer cells and no toxicity to non-cancerous HUVEC cells at similar concentrations (Figure 5). In addition, it is seen that the AgNPs-Car we synthesised are effective in MDA-MB-231 at a 2.4 times lower concentration than Car alone and 5.7 times lower in 4T1 (Table 2). As a result, AgNPs-Car was found to have anticancer effects on treatment-resistant TNBC cells and non-toxic in non-cancerous HUVEC cells (Table 2). In addition, our results show that AgNPs-Car is much more effective on the murine cancer cell 4T1 than the human cancer cell MDA-MB-231. These Our results displayed that higher doses may be required to treat more complex human organisms.

**Table 2:** The cytotoxic effect of Car and AgNPs-Car on MDA-MB-231, 4T1, and HUVEC

| Cell Lines | *$IC_{50}$ (µg/mL) | | |
|---|---|---|---|
| | Car | AgNPs-Car | AgNPs |
| MDA-MB-231 | 69.381 | 28.803 | Not effective |
| 4T1 | 43.244 | 7.580 | Not effective |
| HUVEC | >70 | >70 | Not effective |

Proliferation was examined using an MTT assay for 72 h. * $IC_{50}$: Concentration that inhibited cell growth by 50%. HUVEC: Human umbilical vein endothelial cells, MDA-MB-231: Human breast adenocarcinoma, 4T1: Mouse breast cancer, stage IV, Car: Carmofur, AgNPs: Silver nanoparticles, AgNPs-Car: carmofur-loaded silver nanoparticles

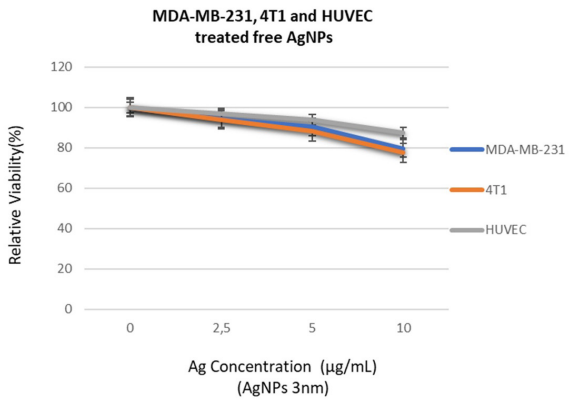


**Figure 6:** Vitality (%) of MDA-MB-231, 4T1 cancerous cells, and HUVEC non-cancerous cells incubated with free AgNPs at various concentrations for 72 h

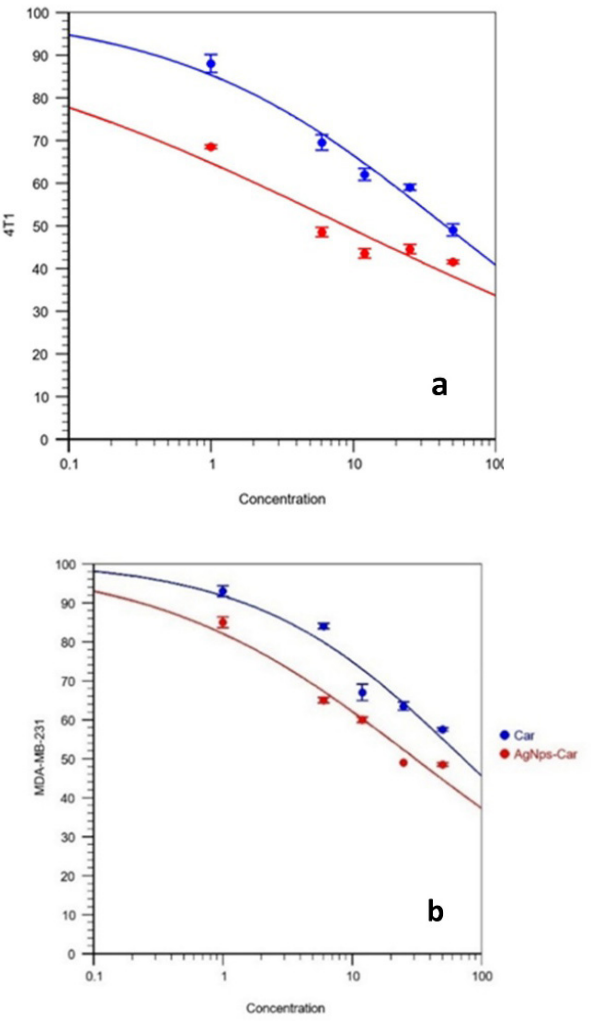


**Figure 7:** Logarithmic $IC_{50}$ curves of Car and AgNPs-Car on 4T1 cells **(a)** and MDA-MB-231 **(b)** cells. *$IC_{50}$ values were determined using the AAT Bioquest $IC_{50}$ calculator

Figure 7 shows that Car and AgNPs-Car had no significant toxicity in normal HUVEC cells. HUVEC lines were interconnected and adhered to the surface in almost all treatment groups. Figure 8 illustrates the morphological differences in MDA-MB-231 and 4T1 cells after incubation in Car and AgNPs-Car at $IC_{50}$ concentrations and HUVEC cells at 50 µg/mL. After the application, the connections between 4T1 cells appeared to be significantly visibly decreased; intercellular links disappeared, cell integrity was affected, and the shape of the cells shrunk compared with the untreated cells. The number of cells and intercellular connections decreased, and some cells became round and detached from the surface in the MDA-MB-231 cells. The investigation of the effect of drug-free AgNPs showed no change or damage in the cells compared with the control group cells. It is also noteworthy that when Car and AgNPs-Car are administered to TNBC cancer

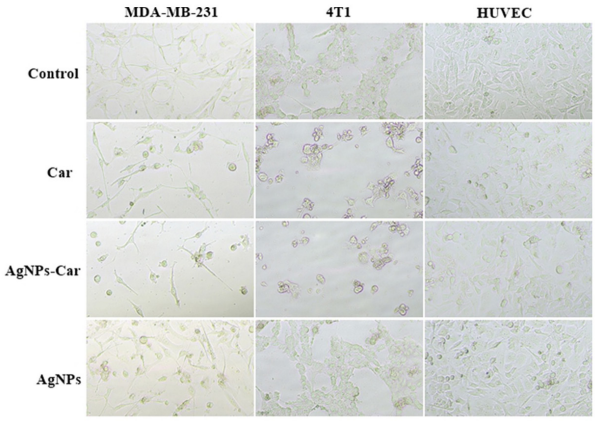


**Figure 8:** Light microscopy of the MDA-MB-231, 4T1, and HUVEC lines. The cells were incubated with Car and AgNPs-Car, AgNPs for 72 h. Magnification: X100. Car and AgNPs-Car were applied to cancerous MDA-MB-231 and 4T1 cells at $IC_{50}$ concentrations, as shown in Table 1, and to non-cancerous HUVEC cells at 50 µg/mL

cells, the cell confluency significantly reduces (Figure 8). The number of TNBC cells was remarkably reduced compared with the number of cells in the control group.

## DISCUSSION

Triple-negative breast cancer may initially respond to chemotherapy, but it can develop resistance over time. These resistance mechanisms are quite complex and are associated with various biological processes, such as the presence of cancer stem cells, activation of DNA repair mechanisms in TNBCs, epithelial-mesenchymal transition (EMT), and the overexpression of drug efflux pumps like P-gp (33). For these reasons, the treatment of TNBC is complex, and in addition to chemotherapy, new therapeutic strategies are being developed, including immunotherapy and targeted approaches. AgNPs represent a promising new approach to the treatment of breast cancer (32). This nanotechnology-based therapeutic method is particularly noteworthy for its potential to specifically target cancer cells and its reduced side effect profile.

Nanoparticle characterisation is the process of determining the physical, chemical, and biological properties of nanoparticles. The synthesis and characterisation of nanoparticles to ensure biocompatibility directly affect their application success. In this study, AgNPs were characterised using SEM, FTIR, DLS, and EDX analyses (34). FTIR spectral analysis of AgNPs and AgNPs-Car was carried out to investigate the changes in the chemical composition of Car after loading into the AgNPs. According to the EDX data, the structure of Car is known to contain elements like carbon, nitrogen, and oxygen, confirming that we were effective in binding Car to AgNPs. SEM automatically analyzes and classifies the particles in the image while providing high-resolution electron imaging. This depends on several additional factors in addition to their size and form, including the particles' surface, volume, convexity, and circularity.

In this study, the AgNPs were captured as highly dense. This is because clumps may occur when we drop the sample liquids on the silicone-based material. It is a natural outcome. Because of this clustering, the particles were seen as more prominent in the SEM analysis (35). DLS is the most commonly used for nanoparticle characterisation among the many techniques because of its fast and easy-to-operate methods and sensitivity to small and low-scattering particles (36). The sizes of the nanoparticles are significant because they influence their action and *in vivo* drug circulation (37). Small sizes of nanocarriers facilitate the drug to pass through the plasmalemma and keep away from recognition and degradation by the reticuloendothelial system (RES) and increase the drug circulation half-time (38-40). However, NPs smaller than 10 nm are not preferred in treatment to avoid being eliminated by fast renal clearance. In addition, NPs larger than 100 nm may not reach the tumour site and may be caught by tissue macrophages (41). The nanoparticles we synthesised are small, which is advantageous in cancer treatment (18, 42). In this study, Car decreased the AgNP size ideally to 10 nm. In addition, nanoparticle size plays a significant role in nanoparticle activity (21-24). The synthesised nanoparticles' ideal sizes for drug delivery systems are thus demonstrated. Our results indicate that the AgNPs-Car induced toxicity predominantly towards treatment-resistant TNBC cells, not non-cancerous normal HUVEC cells. These findings show that AgNPs-Car is effective at several times lower concentrations than the Car, specific to breast cancer cells that are more malignant and non-responsive to drugs.

Treatment with AgNPs is known to cause dose-related toxicity, involving the enhancement of reactive oxygen species and insult of DNA, which cannot maintain cell survival (43). In the studies of Swanner et al., AgNPs have been reported to be toxic in increasing concentrations (5, 10, 20, 40 µg/mL) and in different sizes in MDA-MB-231 TNBC cells (44). For TNBCs, AgNPs with 40 µg/mL of silver have been reported to cause higher toxicity, whereas AgNPs containing 5 µg/mL of silver have lower toxicity. The concentration of silver in the AgNPs is critical; the amount of silver is directly proportional to the toxicity, and AgNPs containing 40 µg/mL of silver have been shown to have the highest toxicity. Also, in the same studies, AgNPs were non-toxic in non-cancerous breast cancer cells (44). Although our findings are similar to the results of Swanner et al., the free AgNPs we synthesised appear less toxic than those in the study (44).

Car, a derivative of 5FU, is primarily used for gastrointestinal cancers and some other solid tumours, though it is still being studied for its effects on breast cancer. A preclinical

study has shown that Car can reduce the metastatic potential of breast cancer cells (45). Therefore, this could represent a significant advancement, particularly for aggressive types of breast cancer such as TNBC. Hence, this study used Car-loaded AgNPs, and their efficacy on TNBCs was evaluated. Nevertheless, researchers have shown that Car and its analogs have an anticancer effect non-selectively (even non-cancerous cells) in some studies; however, the number of studies on Car is limited in the literature (10, 11, 46, 47). In a study, Car, an acid ceramidase inhibitor, was used in treating paediatric brain tumours and was highly effective (4.6-50 μM). It was reported that it could be recommended as a new drug for these tumours (46). Morimoto and Koh noted that the adjuvant use of Car is beneficial in early breast cancer (10). N-acylsphingosine amidohydrolase 1 (ASAH1), an enzyme involved in ceramide metabolism, has been shown to regulate ceramide levels and decrease tumour growth by causing cell cycle arrest and apoptosis (48). According to a study, ASAH1 controls the growth and progression of TNBC by modifying mitogen-activated protein kinase (MAPK). Pharmacological targeting of ASAH1 (through the ceramidase inhibitor carmofur) significantly inhibits the growth of TNBC (49). Furthermore, studies on the NPs-Car are very scarce, and no studies were available for AgNPs-Car (50). Thus, AgNPs synthesised with Car have not been evaluated in TNBCs or cancer treatment. Therefore, our findings are essential to the literature. Our results show that AgNPs-Car created a predominant and selective anti-growth effect on treatment-resistant TNBC cells. Our study was evaluated *in vitro* and was limited by the fact that the biodistribution, biocompatibility, and therapeutic efficacy of AgNPs-Car were not investigated in *in vivo* models. In addition, the long-term stability and cellular uptake mechanisms of the nanoparticles were not investigated in detail. In our study, only cell viability was investigated using the MTT assay, and the mechanisms of cell death such as apoptosis and necrosis were not determined. Finally, further large-scale experiments are needed to comprehensively compare the selectivity in different cancer cell lines and healthy cells. In view of this, further studies are required to evaluate the therapeutic potential of AgNPs-Car.

## CONCLUSION

TNBC is a challenging cancer to treat due to its high heterogeneity, lack of hormone receptors, and aggressive metastatic potential. Current treatments are often insufficient, with no targeted therapy available. In this study, silver nanoparticles conjugated with carmofur (AgNPs-Car) were synthesised and demonstrated strong anticancer effects on resistant TNBC cell lines. Even at low concentrations, AgNPs-Car was effective without harming normal cells, highlighting its potential as a selective and safe treatment. This nanoplatform offers a promising approach for overcoming chemoresistance in TNBC.

**Ethics Committee Approval:** Since the commercially purchased cell lines were used in the study, ethics committee approval was not required.

**Peer Review:** Externally peer-reviewed.

**Author Contributions:** Conception/Design of Study- F.D.K.; Data Acquisition- F.D.K., İ.A.K.; Data Analysis/Interpretation – D.S.K., İ.A.K., F.D.K.; Drafting Manuscript- D.Ö., F.D.K.; Critical Revision of Manuscript- D.Ö.; Final Approval and Accountability- D.Ö., F.D.K.; Technical or Material Support- İ.A.K., D.S.K.; Supervision- D.S.K.

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