# Cytotoxic Activity Evaluation on Breast Cells of Guest-host Complexes Containing Artemisinin

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*Artemisinin is a sesquiterpene lactone with vastly proved anti-cancer effects and a low toxicity profile. However, the compound has poor water solubility, bioavailability and a short half-life. As such, the present paper aims to evaluate the cytotoxic effect on breast cells of three guest-host inclusion complexes containing artemisinin as the active compound and different cyclodextrins as hosts. These were tested using two different concentrations (i.e. 12.5 μM and 25 μM) and three cell lines, namely two human breast adenocarcinoma cell lines (MCF7 and MDA-MB-231) and one human non-tumorigenic breast epithelial cell line (MCF10A) employing the colorimetric microculture tetrazolium assay. After a 72h stimulation period, the most promising results were obtained for the complex containing artemisinin and Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, the cell viability decrease being significant for the estrogen positive MCF7 cell line (80.0 ± 2.3 %), making the complex a potential candidate for further in vivo testing.*

*Keywords: artemisinin, cyclodextrins, breast cancer, cytotoxicity*

Breast cancer, the most common cancer type diagnosed in women, presents with a yearly increase in incidence. Despite this, the prognosis for early diagnosed and properly treated cases has been much better in the last decades, the survival rates increasing considerably [1]. However, standard breast cancer treatments, such as systemic chemotherapy, radiotherapy or antihormonal treatments have long-term direct or indirect adverse effects on the cardiac, nervous and reproductive system, alongside the fatigue, pain, anxiety or emesis experienced during the treatment course [2]. For most standard chemotherapeutic agents, the significant side effects are due to their low specificity. Because of this, it is only natural that scientists are currently investigating alternative compounds that demonstrate not only a good cytotoxic activity on cancer cells, but also low toxic effects on healthy cells [3].

Artemisinin (ART- fig. 1.), a sesquiterpene lactone extracted from Artemisia annua, was proven unique to this species and inexistent in other tested Artemisia species. Depending on the plant's origin and cultivation region, ART's concentration may vary between 0.00095-1.54%, the highest ones being found in the leaves and aerial parts of A. annua [4].

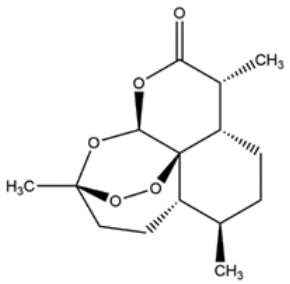


Fig. 1. Chemical structure of ART

The role of artemisinin and its derivatives as anti-cancer agents has become an intensely researched topic in the last decades. Although the first therapeutic target of ART was malaria, many research groups worldwide have proven its potential cytotoxic effect against a series of cancer cell lines [5-7]. Alongside its antiproliferative [8], proapoptotic [9], antimetastatic [10] and antiangiogenic [11] properties, one of the biggest advantages of ART is represented by its lack of severe adverse effects [12-14].

The potential of ART as a cytotoxic agent for breast cancer cell lines was briefly examined by Zheng G. et.al. in 1994 when an analysis on several tumor producing cell lines was published. A human breast adenocarcinoma cell line, namely MCF-7, was included in the study, the presented effective dose (ED50) being over 10μg/mL [15]. Sundar S. et.al. proved in 2008 that when a concentration of 300μM was used, ART downregulated the levels of estrogen receptor $\alpha$ protein after 48 h in the MCF-7 cell line [8]. A highly estrogen responsive cell line, MCF-7 is able to express both $\alpha$ and $\beta$ estrogen receptors, the first type being corelated with high levels of cell proliferation, while the β type is believed to be linked to the inhibition of the tumorigenesis process [16,17].

For an active pharmaceutical ingredient to become a potential drug candidate, the pharmacokinetic properties of the molecule need to be modified as to ensure a high bioavailability for the desired administration route. ART's profile, however, indicates that its low water solubility (0.048 g/L at 37°C [18]) and a significant hepatic first pass effect determine a reduced bioavailability with a short half-life when orally administered [19].

The improvement of these properties can be achieved using cyclodextrins (CDs) as carrier materials. Glucose based macrocyclic structures, CDs present as truncated cones with an internal nonpolar cavity and a hydrophilic exterior. Because of these properties, CDs are able to form guest-host inclusion complexes in which, lipophilic molecules, such as ART, are entrapped inside the internal cavity [20]. The formation process does not include covalent bond formation, the drug molecules found inside the cavity being in equilibrium with free ones. The obtained complex is therefore able to dissociate easily in an aqueous medium [21], to increase the extent of substance dissolution and, as such, to improve oral bioavailability [22], while at the same time enhancing the stability of the pharmaceutical active ingredient [23].

Because of these aspects, the present study aims to evaluate the cytotoxic effect of ART and three inclusion

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complexes prepared using cyclodextrins as guest molecules on two human breast adenocarcinoma cell lines, namely MCF7 and MDA-MB-231 and one human non-tumorigenic breast epithelial cell line, MCF10A. The main goal was to determine both the cytotoxic effect on breast cancer cells vs. healthy cells and the potential of the compounds as anti-cancer agents influenced by the presence or absence of estrogen receptors on the cancer cell lines.

## Experimental part

### *Materials and methods*

Samples

Artemisinin (ART) was purchased from Sigma with a 98% purity and was used without any modifications or purification. The three cyclodextrins used for the formation of inclusion complexes, namely randomly methylated-β-cyclodextrin (CD 1), Heptakis(2,6-di-O-methyl)-β-cyclodextrin (CD 2) and Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (CD 3) were purchased from CycloLab R&D Ltd. (Budapest, Hungary) and were used as received. The solvent used for the preparation of the complexes, ethanol, was obtained from Sigma-Aldrich and presented with the required standard analytical grade.

*Cell culture*

MCF7 – human breast adenocarcinoma cell line (ATCC® HTB-22TM), MDA-MB-231 - human breast adenocarcinoma cell line (ATCC® HTB-26TM) and MCF10A – human breast epithelial cells (ATCC® CRL-10317™) were acquired from the American Type Culture Collection (ATCC). MCF7 cells were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC) and MDA-MB-231 cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich). Each cell line was supplemented with 10% fetal bovine serum (FBS; Gibco, ThermoFisher Scientific) and 1% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/mL; Sigma-Aldrich). MCF10A cells were cultured in Dulbecco's Modified Eagle's Medium F-12 (DMEM:F-12; ATCC) supplemented with 20 ng/mL epithelial growth factor (EGF), 0.01 ng/mL insulin, 500 mg/mL hydrocortisone, 5% FCS and 1% Pen/Strep mixture. Standard conditions were used for cell culture, namely 37°C and a humidified atmosphere containing 5% $CO_2$.

*Preparation of guest-host inclusion complexes*

Using the kneading method, three inclusion complexes were prepared [24]. ART and each cyclodextrin, CD 1, CD 2 and CD 3 were weighted as to obtain a 1:1 molar ratio. In the presence of ethanol, the compounds were mixed in an agar mortar as to form a homogenous paste that was kneaded for 1 h, adding small amounts of ethanol to keep a suitable consistency. The obtained products were dried at room temperature and then 1 h at 40 °C, the final inclusion complexes being brought to a desired form by pulverization.

*MTT assay*

The colorimetric microculture tetrazolium assay (MTT) was used to study the viability of tumor and non-tumor cell lines. The cells were seeded in 96-well culture plates at a cellular density of 1×104 cells/well and allowed to attach to the bottom of the well. The cells were treated with different concentrations of the tested compounds -12.5 and 25 μM (dissolved in dimethyl sulfoxide -DMSO; Sigma-Aldrich) and incubated for 72h. The control group is represented by cells treated with DMSO - the solvent used for sample preparation.The cells were then assayed by the addition of 10μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution from the MTT-based in vitro toxicology assay kit (Sigma-Aldrich). The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 3 h contact period. The precipitated crystals were dissolved in 100μL of lysis solution provided by the manufacturer. Finally, the reduced MTT was spectrophotometrically analyzed at 570, using a microplate reader (xMark Microplate Spectrophotometer, Bio-Rad). All experiments were performed in triplicate.

*Statistical analysis*

The results were expressed as mean ± standard deviation (SD). Comparison among the groups was performed using the one-way ANOVA test followed by Dunnett's multiple comparison test. A p value $\leq 0.05$ was considered to be of statistical significance. GraphPad Prism 5 was used for the statistical analysis. Data presented as mean ± SD was expressed vs. Control in all graphical representations.

## Results and discussions

The formation of the three inclusion complexes using the presented preparation method was analyzed and proved previously by our research team employing thermal analysis and FTIR spectroscopy. An in vitro cell viability assay on A375 and HaCaT cell lines was also presented alongside with the antioxidant activity [24].

The present paper aimed to evaluate the cytotoxic effect of the prepared complexes on breast cancer. In order to determine the effects on cell viability of the cyclodextrin complexes (CPX 1: ART and CD 1, CPX 2: ART and CD 2 and CPX 3: ART and CD 3), different cell lines were used, namely tumor - MCF7 and MDA-MB-231 (human breast adenocarcinoma) and non-tumor MCF10A (breast epithelial cells).

Figure 2 presents the effect of the tested samples on MCF7 human breast adenocarcinoma cells. At the tested doses, namely 12.5 and 25μM, ART induced a mild viability reduction for the tested cells, namely 88.2 ± 11.4 % and 79.9 ± 1.7 %, respectively. The guest-host complexes CPX 1 and CPX 2 did not affect significantly the viability of the cells, but CPX 3 elicited a dose-dependent reduction of tumor breast cells viability, 81.7 ± 3.7 % and 80.0 ± 2.3 %, respectively. In the cases of the pure cyclodextrins, the cells' viability decrease is relatively mild, but influenced by the used concentration. However, from the three tested CDs, CD 3 seems to manifest the highest inhibition effect, with a percentage of cell viability equal to 91.4 ± 3.9 % after 72 h and using a 25μM concentration.

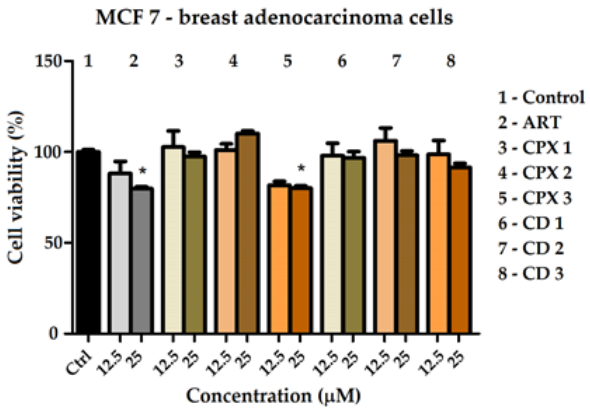


Fig. 2. The in vitro effects of the tested compounds (12.5 and 25 μM) on MCF7 - human breast adenocarcinoma cell line (estrogen receptor +) at 72 h post-stimulation

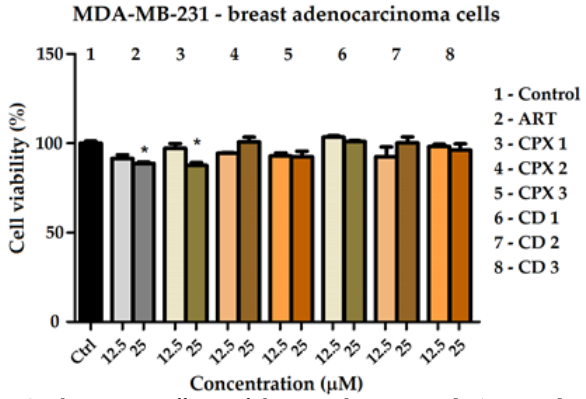


Fig. 3. The in vitro effects of the tested compounds (12.5 and 25 μM) on MDA-MB-231 - human breast adenocarcinoma cell line (estrogen receptor -) at 72 h post-stimulation

Regarding the results obtained for the pure compounds and the prepared inclusion complexes on MDA-MB-231 human breast adenocarcinoma cells (fig. 3), it can be observed that ART decreased in a dose-dependent manner the viability of the cells (at the higher dose tested, namely 25 μM, tumor cells' viability was 88.8 ± 1.3 %). The complex CPX 1 determined a decrease of the cells' viability, especially at the highest dose tested (at 25 μM cells viability being 87.7 ± 2.7 %). For CPX 2 and CPX 3 the cytotoxic effect being slight to almost non-existent, in both cases the values obtained for cell viability being higher than those determined for pure ART. The tested CDs did not alter significantly tumor cells' viability.

On the non-tumor cell line (fig. 4), MCF10A human breast epithelial cells, the determined viability was higher after stimulation with ART compared to the data obtained in the case of both tumor cell lines, namely 94.8 ± 6.3 % at a concentration of 12.5 μM and 93.1 ± 7.6 % at 25 μM, indicating that the compound has a slightly selective effect towards cancer cells. The cyclodextrin complexes induced a mild cytotoxic effect at the higher dose tested, i.e. 25 μM, CPX 2 and CPX 3 displaying similar results (CPX 2 – cell viability 88.4 ± 6.8 % and CPX 3 -cell viability 90.0 ± 8.2 %), while for CPX 1 the viability of the cells was 98.4 ± 2.9 %. In respect to the effect of the CDs on the MCF10A cell line, at a concentration of 25μM, CD 2 elicited a significant decrease in cell viability, namely 73.2 ± 1.0 %.

Artemisinin has been proven to present antiproliferative effects on breast cancer cell lines. A comprehensive study presented by Tin A. et al. concluded that the best in vitro experimental conditions in which the proliferation of two cell lines (namely MCF7 and MDA-MB-231) is inhibited consist of a concentration of 300 μM and a 48h incubation period. Moreover, it was demonstrated that ART is able to

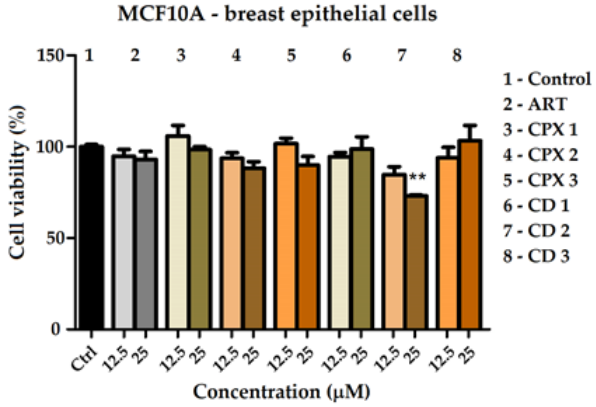


Fig. 4. The in vitro effects of the tested compounds (12.5 and 25 μM) on MCF10A – human breast epithelial cells at 72 h post-stimulation

inhibit the growth of tumor xenografts derived from MCF7 cells and developed in vivo in nude athymic NIH III mice, when injected with 100 mg/kg/day ART [8,25].

In the present study, we aimed to evaluate the cytotoxic effect of ART and three guest-host complexes formed between the active substance and different CDs on two breast cancer cell lines, namely MCF7 and MDA-MB-231 and on one non-tumorigenic cell line, MCF10A. Since breast cancer treatment is modulated according to tumor type and to the estrogen receptor (ER) expression [26], we focused our present study on two different kinds of cancer cell lines. These included an estrogen receptor positive epithelial cell line, MCF7, and an estrogen negative epithelial, highly aggressive and invasive human breast cell line, MDA-MB-231.

Because ART's significant cytotoxic activity was proved on both cell lines at high concentrations [25], we aimed to determine if at lower concentrations (i.e. 12.5 μM and 25 μM), the antiproliferative effects manifest differently between the ER + and ER -cell lines. Alongside with pure ART, the cancer cell lines were also treated with the three guest-host inclusion complexes (CPX 1, CPX 2 and CPX 3) and the pure CDs (CD1, CD 2 and CD 3).

In the case of pure ART, the cytotoxic effect was dose-dependent on both cell lines, with mild differences regarding cell viability of MCF7 and MDA-MB-231 cells. These findings agree with published literature, a study published by Kumari K. et.al. revealing that ART determined cell growth inhibition on both cell lines, alongside decreased mobility for MCF7 cells treated with ART and significantly reduced invasion ability for artemisinin treated MDA-MB-231 cells [27].

Regarding the pure cyclodextrins it can be affirmed that CD 1 and CD 2 didn't influence in a significant manner the cells' viability on neither tested breast cancer cell line, while for CD 3 a slight decrease in viability was observed at the higher concentration tested for the MCF7 cell line (approximately 91 % vs. Control). This was to be expected since a slight cytotoxic effect manifested by methyl-β-cyclodextrin that increases in time and with the concentration was proven by Upadhyay A. et.al. [28].

In the formation of guest-host inclusion complexes the nonpolar molecule enters the internal lipophilic cavity of the CDs and thus, the carrier molecules are widely used as to improve drug solubility and stability [21]. Because the methylation of hydroxyl groups can enhance the hydrophobic cavity, methylated CDs are used in drug delivery processes to improve bioavailability and cellular distribution [29,30], especially Heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin which is considered the most soluble in comparison to natural CDs [31].

Upadhyay A. et.al. demonstrated that MCF7 and MDA-MB-231 pretreated with methyl-β-cyclodextrin proved to be more susceptible to the anti-cancer agent and presented a decreased cell viability than the cells that didn't undergo the pretreatment [28]. An enhancement in cycle cell arrest and apoptosis induction of tamoxifen on melanoma cell lines and of doxorubicin on breast and hepatocellular cancer when associated with methyl-β-cyclodextrin was also demonstrated by Mohammad N. et.al. [32,33]. The enhancement of the cytotoxic activity on various cell lines of anti-cancer compounds was proven when the drugs were associated with modified cyclodextrins, namely with Heptakis(2,6-di-O-methyl)-β-cyclodextrin (CD 2) for melanoma cell lines [34] and Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (CD 3) for hepatoblastoma and breast adenocarcinoma cell lines [35]. However, physico-chemical determinations have revealed an important difference between the inclusion complexes of anti-cancer

molecules with the two modified cyclodextrins. CD 2 proved to possess a restricted conformational flexibility, while CD 3 presents much greater conformational freedom, and, as such, an improved guest-host adaptation on complexation [36].

A dose-dependent effect can be observed for both cell lines regarding CPX 1 and CPX 3, however, CPX 2 doesn't appear to influence the viability of the tested cells. At the highest dose tested, CPX 1 had a more significant effect on the estrogen receptor negative cell line (MDA-MB-231), while CPX 3 decreased in a higher percentage the viability of the estrogen receptor positive cell line (MCF7).

Considering the effect of ART on the MCF7 cell line and the literature data regarding not only the ability of CDs to enhance solubility and to improve the cytotoxic activity, but also the presented differences in respect to their guest-host complexation ability, it was only to be expected that CPX 3 (containing ART and Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin) would demonstrate the most significant effect on the tested breast cancer cell lines, this proving superior in the estrogen receptor positive MCF7 cell line.

About the cytotoxic effect of all tested compounds on the human breast epithelial cells MCF10A, it can be affirmed that neither pure ART nor the prepared complex CPX 1 determined a significant decrease in the viability of the healthy cells. ART's lack of toxic activity on normal epithelial breast cells is in agreement with data found in literature [27]. The significant decrease in cells viability determined for CD 2 and CPX 2 on MCF10A cell line coupled with the lack of an important cytotoxic activity on either tested breast cancer cell lines makes this complex inadequate as a potential anti-cancer agent on these cell lines.

Despite the mild decrease in the viability of the MCF10A cells determined by CPX 3, its significant effect on the MCF7 cell line makes this guest-host inclusion complex the most suitable choice of the three prepared complexes.

## Conclusions

Artemisinin's potential as an anti-cancer agent has been vastly evaluated in recent years. Despite the significant cytotoxic activity on several cancer cell lines, breast cancer among them, a fundamental disadvantage regarding ART's water solubility requires the development of new formulations to overcome the poor bioavailability that comes as a direct consequence. As such, the present study aimed to evaluate the cytotoxic activity on both estrogen receptor positive (MCF7) and estrogen receptor negative (MDA-MB-231) cell lines, as well as the influence on healthy epithelial breast cells (MCF10A) of three guest-host inclusion complexes formed between ART and three different cyclodextrins.

The inclusion complex formed between ART and CD 3 (Heptakis(2,3,6-tri-O-methyl)-â-cyclodextrin), namely CPX 3, proved to determine the lowest cell viability percentage. The determined cytotoxic effect was dose-dependent and was more significant than the one observed for the pure compounds tested alone. Even though a mild cytotoxic effect on MCF10A cell line was revealed, the decrease in tumor cell viability was far more relevant, a difference regarding the two types of tested cell lines being observed at the tested concentrations and, as such, CPX 3 proving to be more active on MCF7, the estrogen receptor positive cell line. In conclusion, we assess that this type of formulation could become a potential anti-cancer alternative, after further in vitro evaluations and higher concentrations and in vivo determinations.

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