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Research Article

Anti-melanoma activity of green-produced nanosilver-chlorhexidine complex

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Abstract

This study follows the in vitro anti-melanoma activity of silver nanoparticles (Sn) obtained by "green" reduction with catechins from *Camellia sinensis* and superficially charged with chlorhexidine diacetate (Cx+). The antiproliferative activity was assessed on human keratinocytes (HaCaT) and human melanoma (SH-4) cell lines. The respective IC_{50} values and selective indexes (SI) were retrieved for both the non-conjugated and [Cx+]-conjugated nanosilver forms (Sn-Cx+). As a next step, the active Sn-Cx+ complex was formulated into a prototype of an adhesive patch comprised of ammonio methacrylate copolymer type B and hydroxypropyl methylcellulose in a 1:2 ratio. This resulted in a strong anti-tumor effect (IC_{50} 0.759 ± 0.062 μg/mL) with a high selectivity (SI 4.0) exceeding even the bare colloid's performance that was recorded. The findings of this experiment suggest the potential applicability of the invention in the local chemotherapy of skin melanoma in its early or post-operative stage.

Keywords

silver nanoparticles, chlorhexidine functionalization, skin cancer, green technology, adhesive patch

Introduction

Melanoma is a rapidly progressing skin cancer caused by the abnormal proliferation of melanocytes (Krishnan and Mitragotri 2020; Adnan et al. 2023). In contrast to the slow-developing and locally invasive non-melanoma skin cancers, such as basal and squamous cell carcinomas, melanoma is characterized by a much lower prevalence but a high mortality rate after metastasis (Zeng et al. 2023). Therefore, local chemotherapy is rarely considered a therapeutic alternative (Bei et al. 2010; Niu et al. 2017). Indeed, only stage 0 of progression (in situ melanoma) and the postoperative phase afterward have been an object of investigation in this respect. As a result, imiquimod cream (Aldara®, Zyclara®) has been proven effective in patients with persistently positive margins of melanoma in situ after surgical excision (Fan et al. 2015; Verga et al. 2019; Vaienti et al. 2023). Except for that, there are mostly reports of in vitro or in vivo animal investigations testifying to the potential efficacy of the designed therapeutic formulations in the local melanoma treatment. Among the explored therapeutic agents for the purpose are 5-fluorouracil, dacarbazine, doxorubicin, non-loaded or drug-conjugated metal nanoparticles (Lam et al. 2008; Najar and Dutz 2008; Paolino et al. 2008; Hafeez and Kazmi 2017; Niu et al. 2017; Sahu et al. 2017, 2019; Tambunlertchai et al. 2023).

Silver nanoparticles are now widely recognized as multifunctional tools in nanomedicine, drug delivery, and theranostics (Ivanova et al. 2018; Sakthi et al. 2022). Most distinct are their explicit and wide-spectrum antimicrobial properties and intrinsic anti-tumor activity (Duman et al. 2024; Fahim et al. 2024). Generally, both of these

qualities are highly valued in the design of topical formulations for cancer treatment. Ideally, a local chemo agent should provide selective cytotoxicity against the mutant cells, anti-inflammatory activity, and prevention or cure of an accompanying infection (Newman and Zloza 2017; Voiculescu et al. 2019; Quezada et al. 2020; Zappavigna et al. 2020; von Montfort et al. 2024). Despite the numerous superlatives on behalf of nanosilver, these metal nanoparticles cannot be regarded as a defined therapeutic agent. The various techniques and the many possible reducers applied in their production determine a highly differentiating surface functionality (shape, size, charge, "cap"), which is the key to any pharmacological activity, including toxicity (Menichetti et al. 2023; Veena et al. 2023). So far, silver nanoparticles with positive zeta potential or wire shape have been proven more cytotoxic as compared to negatively charged colloids or spherical clusters, respectively, and this could potentially work in their favor if a selective anticancer agent is sought after (Stoehr et al. 2011; Kim et al. 2013). A size above 10 nm, on the other hand, limits the nuclear penetration and gene toxicity of nanosilver (Ahmed et al. 2017).

This study offers ongoing research on recently obtained conjugates of silver nanoparticles with chlorhexidine (Cx+) (Ivanova et al. 2023, 2024). The antiseptic was chosen as a functionalizing agent with the purpose of achieving a complex nanosilver agent with fortified antimicrobial activity. The nanosilver suspension was obtained by reduction with catechins from *Camellia sinensis* (green tea). Like most silver nanoparticles synthesized through the reduction of silver ions with plant polyphenols, the native colloid was characterized by a negative zeta potential, more precisely $\zeta = -50.01$. The particles were spherical and had an average hydrodynamic diameter of 92.34 nm. Upon conjugation with Cx+, naturally, the colloid acquired a positive charge ($\zeta = +44.59$) from the superficially adsorbed molecules of the drug, enlarged in size (142.5 nm), and most importantly—enhanced microbicidal activity against viruses and bacteria. These properties, along with the highly increased cytotoxicity in the presence of Cx+, provoked interest in further investigations in the direction of the anti-proliferative activity against topically accessible cancers. Melanoma, as an eloquent example of such a tumor, was chosen as a prime target of the current research.

Materials and methods

Materials

Silver nitrate (>99.9%) and sodium hydroxide (>98%) were purchased from Thermo Fisher Scientific, Oxford, UK; chlorhexidine diacetate salt hydrate (≥ 98%, Mw 625.55 g/mol) was purchased from Sigma Aldrich, Burlington, MA, USA; hydroxypropyl methylcellulose (HPMC) (80–120 cps) was supplied by Sigma-Aldrich, St. Louis, MO, USA; ammonio methacrylate copolymer (type B) (Eudragit® RS 100) was a kind gift from Evonik Industries AG, Darmstadt, Germany; all organic solvents were supplied by Sigma-Aldrich, USA, in analytical grade.

The culture reagents were supplied by the following: Dulbecco's modified Eagle's medium (DMEM)—Sigma-Aldrich, Schnelldorf, Germany; fetal bovine serum (FBS)—Gibso/BRL, Grand Island, NY; penicillin and streptomycin—LONZA, Cologne, Germany. Disposable consumables were supplied by Orange Scientific, Braine-l'Alleud, Belgium.

The cell lines—HaCaT (ATCC® № PCS-200-011™; human keratinocytes) and SH-4 (ATCC® № CRL-7724™; human melanoma)—were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Methods

Synthesis and functionalization of silver nanoparticles

The established procedure for the synthesis and conjugation of silver nanoparticles (Sn) in detail is published elsewhere (Ivanova et al. 2023); wherefore, here, it will be communicated only briefly. Silver nanoparticles were obtained by reducing an aqueous silver nitrate 2 mM solution with *Camellia sinensis*-derived phenolic fraction, rich in catechins, and following purification by a dialysis method. The particles were functionalized with chlorhexidine diacetate (Cx+) using a simple procedure of mixing the colloidal and drug solutions in a defined ratio, which led to passive surface adsorption of the antiseptic onto the negatively charged metal surface. The obtained colloidal solutions were standardized to 700 μg/mL nanosilver and 1130 μg/mL Cx+. For clearance, in the results section, all data is expressed as Sn concentration, from which the Cx+ concentration could be calculated by default if needed.

Adhesive patch

The Sn-Cx+ complex was formulated into a model of an adhesive patch (P1) suitable for direct application or in situ film-forming composition. For this purpose, two stock solutions were prepared: 150 mg Eudragit® RS 100 (ERS) in 2.5 mL dichloromethane (DCM) and 300 mg hydroxypropyl methylcellulose (HPMC) in 10 mL ethyl alcohol 95% w/w. To the latter solution was added 1.5 mL of the active Sn-Cx+ colloidal dispersion under continuous stirring (IKA® C-MAG HS 4 magnet stirrer, Staufen, Germany), and this step ensured the complete dissolution of the swelled cellulose polymer. Both organic solutions were homogenized in a hermetically sealed glass tube. Finally, the mixture was enriched with 2.5% v/v glycerol. So obtained, the dispersion was standardized to a final volume of 15 mL with ethanol 95% w/w and cast into Petri dishes with a surface of 44.2 cm^2, each of which received exactly 4.42 mL of evenly spread formulation onto the bottom. The casts were left to vaporize and solidify in a ventilated laboratory hood for 4 h. The active concentration in the patches thus obtained was 70 $\mu g/cm^2$. The prepared samples were stored in a refrigerator before use.

Antiproliferative activity

Test samples

The samples from the native silver nanoparticles suspension (Sn) and the conjugated form of the colloid (Sn-Cx+) were applied in their stock concentrations of 700 μg/mL (expressed as nanosilver).

A sample with a starting concentration of 70 μg/mL was restored from the patch formulation (P1) by hydrating a single piece (44.2 cm^2) with 4.42 mL distilled water. The hydration process was carried out in a closed petri dish until complete swelling and dissolution of the plaster in the liquid. The viscous liquid thus obtained was transferred into an Eppendorf tube and further homogenized on a vortex mixer (model ZX4, Velp Scientifica, Italy). As it will be later a subject of discussion, the respective concentrations of the polymeric excipients ERS and HPMC in the so-prepared sample were 10 mg/mL (1% w/v) and 20 mg/mL (2% w/v).

All samples were subjected to the in vitro test for anti-proliferative activity within 72 h after preparation.

Cell cultures

HaCaT and SH-4 cells were cultured in cell culture flasks with areas of 25 cm^2 or 75 cm^2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and the antibiotics penicillin 100 U/mL and streptomycin 0.1 mg/mL, at 37 °C in a 5% CO_2 atmosphere with 90% relative humidity. While in exponential growth, the cells were seeded in 96-well plates with a defined density of 1×10^3 cells per well in 100 μl DMEM using trypsin cell dissociation. The in vitro experiments were performed after 24 h of culturing of the prepared plates under the above conditions.

Antiproliferative activity

The 24 h-incubated adherent cell monolayers of HaCaT and SH-4 cells were contacted with the test samples in 2-fold dropping dilutions. Neutral Red medium was added to the wells 72 h after incubation, and the cultures were allowed to uptake the dye for another 3 h incubation. Then, the wells were washed with phosphate buffer saline pH 7.4, and a mixture of ethyl alcohol, acetic acid, and distilled water in a 49:1:50 ratio was added. A neutral red uptake in vitro test (NRU assay) was carried out for evaluation of cell viability by measuring the optical density at 540 nm (OD_{540}) on an ELISA microplate reader (TECAN, Sunrise™, Groedig/Salzburg, Austria). The antiproliferative activity (APA) of the test samples was calculated through the equation:

$$\% \text{ (APA)} = [1 - OD_{540} \text{ (test sample)}] / [OD_{540} \text{ (negative control)}] \times 100$$

The 50% inhibitory concentrations (IC_{50}) were determined as the concentrations of the test samples that caused 50% inhibition of the cell proliferation as compared to the untreated (negative) control sample. The selectivity index (SI) was calculated as the ratio of the IC_{50} value on the HaCaT cell line to the IC_{50} value on the tumor SH-4 cell line:

$$SI = IC_{50} \text{ HaCaT}/IC_{50} \text{ SH-4}$$

Statistical analysis

The values were presented as means (± SD) of six repetitions. A t-test was carried out to outline statistically significant ($p < 0.05$) differences between the antiproliferative activity of the test samples.

Results

The antiproliferative activity of Sn and Sn-Cx+ on human keratinocytes (HaCaT) and human melanoma cells (SH-4) was established. The conjugated nanosilver form was found to act as an above 18-fold stronger cell growth inhibitor, judging by the IC_{50} values on both the normal and the mutant cell lines. Most importantly, by applying the Sn-Cx+ complex, a notably higher selectivity (SI = 2.62) was achieved. The concentration-dependent effects of the native and the [Cx+]-charged nanosilver colloid are shown in Fig. 1.

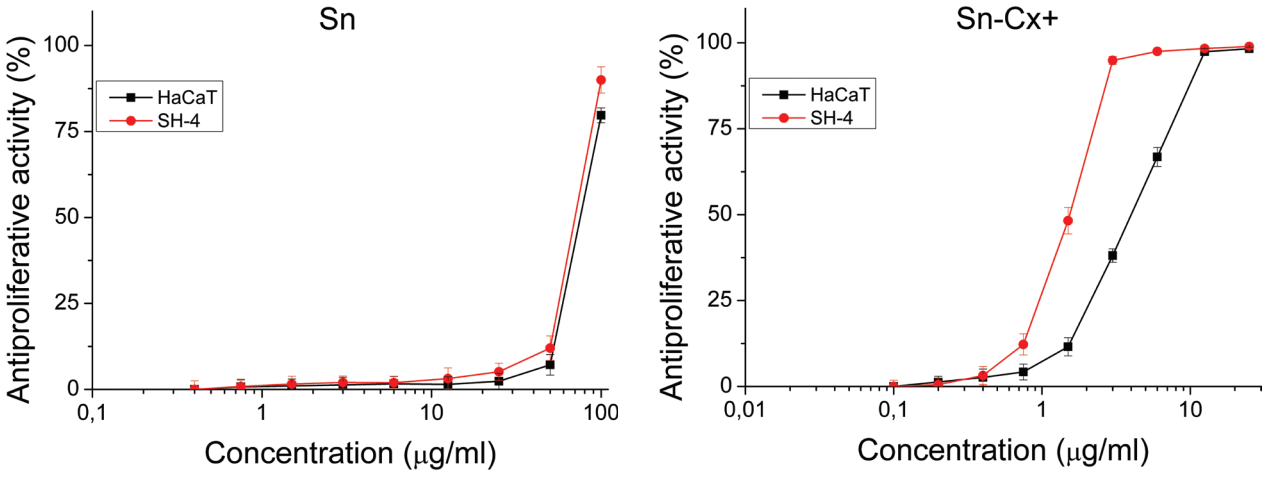

Figure 1. Antiproliferative activity (%) of Sn (left) and Sn-Cx+ (right) on HaCaT and SH-4 cell lines.

The formulated Sn-Cx+ (P1) was found to retain the intrinsic antiproliferative activity of the complex after reconstitution and to even twice decrease the IC_{50} on the tumor SH-4 line. Interestingly, the same effect was observed on the normal HaCaT cell line but to a substantially lesser extent. Hence, a significant increase in the formulation's selectivity (SI = 4.0) was recorded. The results are presented in Fig. 2 and Table 1.

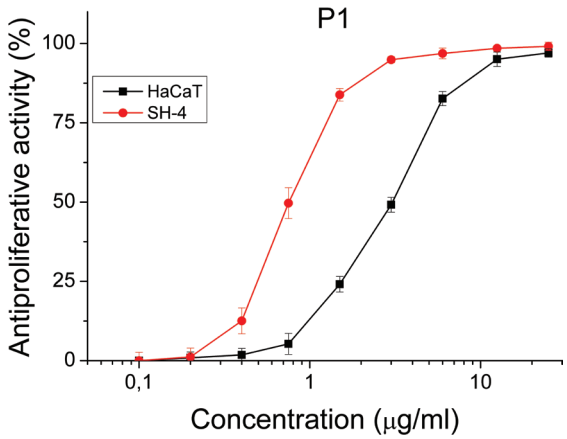

Figure 2. Antiproliferative activity (%) of P1.

Table 1. Antiproliferative activity of Sn, Sn-Cx+, and the formulated active complex (P1).

Sample	Mean IC_{50} ± SD (μg/mL)		SI*
	HaCaT	SH-4	
Sn	75.35 ± 1.36	70.14 ± 1.64	1.07
Sn-Cx+	4.02 ± 0.19	1.54 ± 0.09	2.62
P1	3.037 ± 0.140	0.759 ± 0.062	4.0
p-value	< 0.0001	< 0.0001	n/a

*p-value was calculated for Sn-Cx+ and P1 to follow the significance of the polymeric excipients for the anti-proliferative activity.

Discussion

By any means, the results of this study revealed the explicit and selective anti-melanoma activity of the Sn-Cx+ complex, alone and formulated into a bioadhesive film of ERS and HPMC. First, a distinctive difference was established in the activities of the native and the [Cx+]-conjugated silver nanoparticles. Chlorhexidine, except for being known as a broad-spectrum antimicrobial agent, has been reported to possess anticancer properties on various tumor cell lines (Gräber et al. 2013; Khairnar et al. 2018; Martínez-Pérez et al. 2019). The high cytotoxicity of the compound is determined by its cationic surfactant nature but is, in general, non-specific (Hidalgo and Dominguez 2001; Liu et al. 2018). Focusing on the drug-bearing units, viz., the silver nanoparticles, the drastic shift in the zeta potential to positive values upon conjugation with Cx+ (from −50.01 for Sn to +44.59 for Sn-Cx+) could also be seen as a reasonable reason for the observed enhanced anti-tumor effect (Wypij et al. 2021). The anticancer activity of nanosilver has been an object of thorough discussions and investigations. From what has been established so far, several leading mechanisms of the cytotoxic and antitumor activity could be outlined, and they include cell membrane rupture, endocytosis-mediated cell entry, reactive oxygen species (ROS) generation, subsequent suppression of the mitochondrial function, dysfunction of proteins and enzymes, lactate dehydrogenase leakage, possible nuclear uptake, chromosome aberration, DNA damage, and apoptosis (Chugh et al. 2018; Morais et al. 2020; Carvalho-Silva et al. 2024). A quantitative real-time polymerase chain reaction (PCR) analysis, performed by Subbiah and Beedu (2018), who tested Ag–Au bimetallic nanoparticles on the murine melanoma cell line B16F10, revealed an up-regulation of the apoptotic genes p53, caspase-3, and caspase-9, followed by a down-regulation of the anti-apoptotic genes Bcl-2 and Bcl-x(K). It is interesting to compare the results from this study with the findings of similar investigations concerning the in vitro anti-melanoma activity of nanosilver formulations; expediently, only studies reporting IC_{50} values have been chosen for this purpose (Table 2).

Next, an impression makes the impact of the polymeric excipients ERS and HPMC. Clearly, not only was the antiproliferative activity of the Sn-Cx+ found enhanced in their presence, but also the selectivity with respect to the tumor SH-4 cell line. A viscosity-determined inhibition of cell growth could be excluded since the established active Sn-Cx+ concentrations of <1 mg/mL correspond to a negligible low polymeric content—<0.015% for ERS and

Table 2. Anti-melanoma activity and selectivity of silver nanoparticles from literature.

Active agent	Tumor line(s)/IC_{50}	Normal line/IC_{50}	SI	Reference
Sn-Cx+ (adhesive patch formulation P1)	SH-4/ 0.759 ± 0.062	HaCaT/3.037 ± 0.140	4.0	current study
Myco-synthesized silver nanoparticles with a size within 10–20 nm and ζ +19.9 mV	SKMEL3/ 17.70 μg/mL	n/a	n/a	Himalini et al. 2022
Sucrose-coated silver nanoparticles with an average size of 6.7 ± 3.2 nm and ζ up to −46.62 mV	A375/17.72μg/mL;SKMEL28/IC_{50} 20.99μg/mL;WM35/6.86μg/mL; B16F10/21.04 μg/mL	HSF/ >40 μg/mL; JB6/ >40 μg/mL	>1.9	Kuang et al. 2022
Ginseng berry extract-reduced silver nanoparticles with a size within 10–20 nm and hydrodynamic diameter of 179 nm	B16BL6/IC_{50}, 114.8 μg/mL	HDF/IC_{50}, 148.6 μg/mL	1.29	Jiménez Pérez et al. 2017
Siver–Gold bimetallic nanoparticles with a size within 1–12 nm ± 0.50 and ζ −24.3 mV	B16F10/1.95 μg/L (0.00195 μg/mL)	n/a	n/a	Subbiah and Beedu 2018
Polyvinylpyrrolidone (PVP)-coated silver nanoparticles with an average size of 35 ± 15 nm, hydrodynamic diameter of 70 nm, and ζ −15 mV	B16F10/4.2 μg/mL	n/a	n/a	Valenzuela-Salas et al. 2019

<0.03% for HPMC. However, the cellulose derivate has been shown to improve the tolerability and increase the anti-tumor and antibacterial properties of silver nanoparticles in a dose-dependent manner (Abdellatif et al. 2021; Filimon et al. 2023; Ko et al. 2023). A potential reason in this particular case could be sought in the superficial absorbance of the polymeric molecules onto the nanosilver particles, which directly affects their size (larger size is associated with lower toxicity), charge, release pattern of silver ions, decomposition, and cellular uptake ability (Dong et al. 2014; Borowik et al. 2019; Filimon et al. 2023). HPMC alone, on the other hand, has been proven to not affect the proliferation of HaCaT and SH-4 cells even in much higher concentrations (up to 2% w/v) (Kamenova et al. 2024).

Last, outside the scope of this research, the proposed Sn-Cx+ complex has already been characterized with distinct antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, and some viruses (Ivanova et al. 2023, 2024). This is another reason to believe that the conjugates possess a high potential to serve as a topically active agent in anti-melanoma formulations, especially those designated for post-surgical therapy.

Conclusion

This study revealed an explicit anti-tumor activity of "green"-synthesized silver nanoparticles and chlorhexidine-conjugated silver nanoparticles against human melanoma cell lines. The functionalized Sn-Cx+ form expressed an 18-fold stronger antiproliferative effect and 3 times higher selectivity against the mutant cells as compared to normal human keratinocytes. The so-obtained nanosilver complex was incorporated into a patch formulation comprised of a classic combination of a bioadhesive polymer (HPMC) and a structure-stabilizing polymer (ERS). Although the patch composition was not yet subjected to a biopharmaceutical characterization and was only proposed as a model carrier system, the selected excipients combination showed a remarkable potentiation of the complex's activity and selectivity and thus potential compatibility and applicability in future dosage forms development.

Additional information

Conflict of interest

The author has declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

Use of commercially available immortalised human and animal cell lines: American Type Cultures Collection (ATCC), Rockville, MD, USA: HaCaT (ATCC® № PCS-200-011™; human keratinocytes) SH-4 (ATCC® № CRL-7724™; human melanoma).

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Author contributions

The author solely contributed to this work.

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Data availability

All of the data that support the findings of this study are available in the main text.

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