

Dual Effects of Titania Nanoparticle (TNP) Combination Treatment with a Chemodrug, Cisplatin Targeting for Nasopharyngeal Carcinoma

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Abstract: Standard treatment for progressive nasopharyngeal carcinoma (NPC) is chemo drug cisplatin (CDDP) as the sole treatment or adjuvant with chemoradiation. Integrating CDDP with the nanosystem has become a current research interest to enhance the effectiveness of chemo drug therapy for NPC. Nanoparticles such as titania nanoparticles (TNP) possessed higher adsorption properties into cells remarkably from the large surface area. Present work studied the combination treatment of TNP and CDDP towards nasopharyngeal carcinoma (NPC) cell lines models. The half-maximal inhibitory concentration (IC₅₀) of CDDP and TNP were characterized on NPC cells, respectively. Further optimizations of the CDDP-TNP dual effect were studied, especially on the cytotoxic profile. The combined treatment of CDDP and TNP was more effective than a single treatment at inhibiting NPC cells by more than 60 %. On the other hand, this combined therapy had dual effects and was highly dependent on the dose of TNP exposed after 24-h exposure. This study suggested that the combination treatment of CDDP and TNP on NPC cells would improve the therapeutic outcomes even at lower concentrations of CDDP, thus could overcome the limitation of current cancer therapy in a future application.

Keywords: cisplatin; combination treatment; Epstein-Barr virus; nasopharyngeal carcinoma; titania nanoparticles.

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1. Introduction

Nasopharyngeal carcinoma (NPC) has been reported as one of the top ten types of cancer diagnosed worldwide, contributing to 0.8 % of total death [1]. Chemotherapeutic cisplatin (CDDP) was known as the standard treatment of NPC decades ago. Over time, the current treatment of NPC with CDDP is still attention; however, the drawbacks such as causing the growth resistance cancer cells, damaging surrounding cells, and recurrent cancer post-

therapy [2] required combinative treatment of chemo drugs, radiation, or through the nanomedicine technology with uses of nanomaterials. Yang and co-workers [3] summarised radiation's advantages, limitations, and current progress as NPC treatment. The secluded location of the NPC site was reported as a challenge for this treatment. Therefore, tedious preparation and meticulous invasive practice are needed to avoid damage to the surrounding cell. The delivering chemo drug cisplatin (CDDP) from titania (TiO₂) particle showed improved release activity against NPC tumors of rats, as mentioned in a study by Liu *et al.* [4]. The hybrid of TNP and biomolecules were studied comprehensively due to potential photocatalytic properties, nano-sized TNP, and less toxicity with no report of TNP absorption in the gastrointestinal tract [5]. One of the discussed applications is TNP combination with chemotherapeutic drugs for cancer targeting. Combination treatment is vital in preventing higher concentrations of chemo drugs, which is the main factor in developing resistance to cancer.

Through the combination treatment, the lower dose or the same dose of CDDP can be applied as the idea of synergistic effect with nanomaterial might increase the effectiveness of the treatment. Moreover, the combination treatment also effectively reduces the tumor size, promotes the targeted drug site, and reduces the treatment's undesirable side effects [6]. Few nanoparticles were widely investigated for combination treatment, such as zinc oxide, gold, palladium, and titania nanoparticle (TNP). Introducing nanoparticles with chemo drugs has been continuously studied; however, the use of titania nanoparticles (TNP) was common due to their exceptional properties in the medical field. TNP, in general, has anti-cancer properties and own exceptional size and biocompatibility properties [7,8]. Combination treatment of TNP and the chemotherapeutic drug had been suggested to be effective for cancer therapy through synergistic reaction [9]. Besides, the outside force was needed to activate the synergistic effect in most cases, such as sonodynamic therapy and solvent deposition method [10], to load the drug into the TNP. However, a more straightforward method is also applicable, as demonstrated by Babitha & Korrapati [11] through the immersion and stirring method, which was also conducted in our work.

2. Materials and Methods

2.1. Materials.

Two types of NPC cells were used in this work: recurrent NPC (NPC/HK-1) and Epstein-Barr virus (EBV-related NPC), C666-1. NPC/HK-1 was obtained from Professor George Tsao from the University of Hong Kong. Meanwhile, C666-1 was a gift from Profesor Kwok-Wai Lo from the Department of Anatomical & Cellular Pathology, Faculty of Medicine, The Chinese University of Hong Kong. SH-SY5Y (ATCC® No. CRL-2266™) and HEK 293 (ATCC CRL-1573) were revived and maintained in complete DMEM under the same culture condition. Anatase TNP, p25 with purity > 99.5 %, was purchased from Sigma-Aldrich (Cas No: 13463-67-7). Cisplatin (CDDP), a chemo drug used in this work, was obtained from the Department of Clinical Pharmacy of Hospital Universiti Sains Malaysia (HUSM@Bertam) with chemical handling was critically supervised by the department and following the protocol of chemo drug handling.

2.2. Sample preparation.

The white powder was weighed at 10 mg and diluted in culture media of 2 mL to prepare 5 mg/mL dilution. The mixture was properly mixed by sonication as adapted from Estrada-Monje *et al.* [12].

2.3. Optimization on preparation protocol for TNP.

Optimization on the method to introduce TNP to cell culture had been performed due to a few conflicting methods; dispersion and sonication before treatment [13,14]; meanwhile, Garcia-Contreras *et al.* [15] used supernatant from sonicated TNP for treatment. The prepared TNP was filtered through the filtration method in a 0.22 µm size filter, resulting in a clear suspension liquid. Meanwhile, unfiltered samples appeared to be cloudy. Next, another group of samples prepared through centrifugation to disperse the particles were applied at 1000 rpm, 5 mins. The clear suspension was taken out and introduced to cells. The two selected concentrations were used for each method. Statistical analysis of One-Way ANOVA by SPSS 26.0 was applied for data analysis.

2.4. Cell viability analysis.

The method of preparing the mixture of TNP-CDDP was proposed according to Salama *et al.* [16]. Briefly, the NPC cells were treated with TNP at concentrations of 5, 10, 50, 100, 250, 500, and 1000 µg/mL). In our work, the TNP had been introduced at increasing concentration. Half-maximal inhibitory concentration (IC₅₀) of CDDP treatment of NPC cells was determined in a separate experiment. Subsequently, the two optimized concentrations of TNP were selected and mixed with CDDP at IC₅₀ value, respectively, for each NPC/HK-1 and C666-1. The cells were exposed to the combination treatment for 24-h. The cell viability kit from Promega (USA), CellTiter 96® Aqueous One Solution Cell Proliferation Assay, was used to measure the absorbance of viability at 490 nm through a microplate spectrophotometer reader (PowerWave™ BioTek, USA).

2.5. Statistical analysis.

All data were expressed in mean ± SD with data normalized to control (untreated cell). Data were statistically analyzed by SPSS 26.0 of One-Way ANOVA (Bonferroni).

3. Results and Discussion

3.1. Preparation of TNP dilution.

The result of the optimization showed the unfiltered mixture of TNP in culture media to be the most effective method of preparing TNP for treatment. However, this method appears to be only applicable at low concentration (100 µg/mL), while at high concentration (1000 µg/mL), the mixture becomes too thick and unable to be pipetted. On the other hand, filter samples might cause loss of NP in the media which is seen through the clear appearance of the mixture after filtration. Application of the supernatant of TNP mixture was expected to be the best method of TNP introduction in cancer therapy; however, the result in Figure 1 showed the opposite outcome. No difference in cell inhibition observed might be suggested the supernatant has no TNP; therefore, no killing effect observed compared to other methods. The statistical

analysis between the groups was analyzed. One-way ANOVA showed no significant difference between filtered 100 and 1000 µg/mL except for filtered 1000 µg/mL and unfiltered of the same concentration ($p = 0.007$). Moreover, another statistical difference was observed for the group unfiltered of both concentrations ($p = 0.002$).

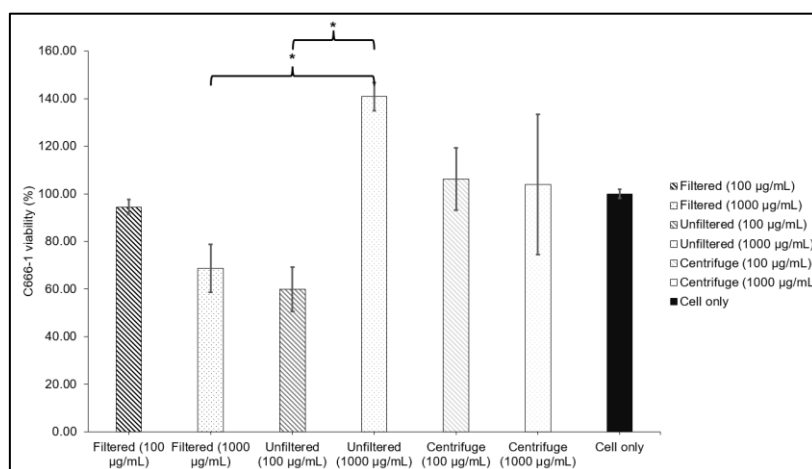


Figure 1. Comparison of different methods for the preparation of TNP for *in vitro* work. The TNP at 1000 µg/mL of unfiltered one showed the highest cell viability with 125.43 % suggested agglomeration of nanoparticles on the cell surface, thus interfering with the absorbance reading, $p < 0.05$. Data were expressed in mean $\pm$ SD, $n = 3$.

Data in Figure 2 demonstrated the viability curve of NPC, SH-SY5Y, and HEK 293 upon interaction with TNP at various concentrations to obtain the optimum concentration for combination treatment. The outcome presented in Figure 2 (a – d) showed various findings on the TNP effectiveness in inhibiting cell proliferation. Compared to other cells, only C666-1 displayed an inhibitory effect, with the lowest recorded at 55.91 % after 100 µg/mL treatment. NPC/HK-1 displayed only 5.32 % inhibition; meanwhile, SH-SY5Y and HEK 293 showed 101.75 % and 91.08 % viability, respectively. Statistically, all data presented with no significant effect of TNP on the studied cells ($p = 1.000$). Further work on combination treatment was proceeding at 100, and 1000 µg/mL on NPC cells only as a killing effect was observed on these cells with data on C666-1 and literature as references.

3.2. Cells viability with TNP.

Microscopy analysis of the C666-1 cells was displayed in Figure 3 (a – j). The presence of TNP was observed from the image below, especially at concentrations of 100 and 1000 µg/mL noted with the black spot scattered around the well like in Figure 3 (b, c, g, and h). The cell inhibition effect was seen when the cells were exposed to CDDP. Subsequently, when the cells were treated with the mixture of CDDP and TNP, the killing effect was expected and demonstrated by the changes of the cell morphology compared to control. As seen in Figure 3, the cells shrank and became lesser than the cells treated with the presence of the CDDP compared to when exposed to TNP only. Combination treatment of TNP at 100 µg/mL and 1000 µg/mL with CDDP at IC_{50} concentration (0.50 mM) was studied for 24-h. Analysis on a combination of TNP and CDDP was successfully demonstrated 40.92 % and 45.31 % viability of C666-1, showing the effectiveness of treatment on the EBV-related NPC (Figure 4). However, notably, the inhibition presented showed no significant difference ($p = 1.000$) for all groups of samples after normalized with control (untreated cell).

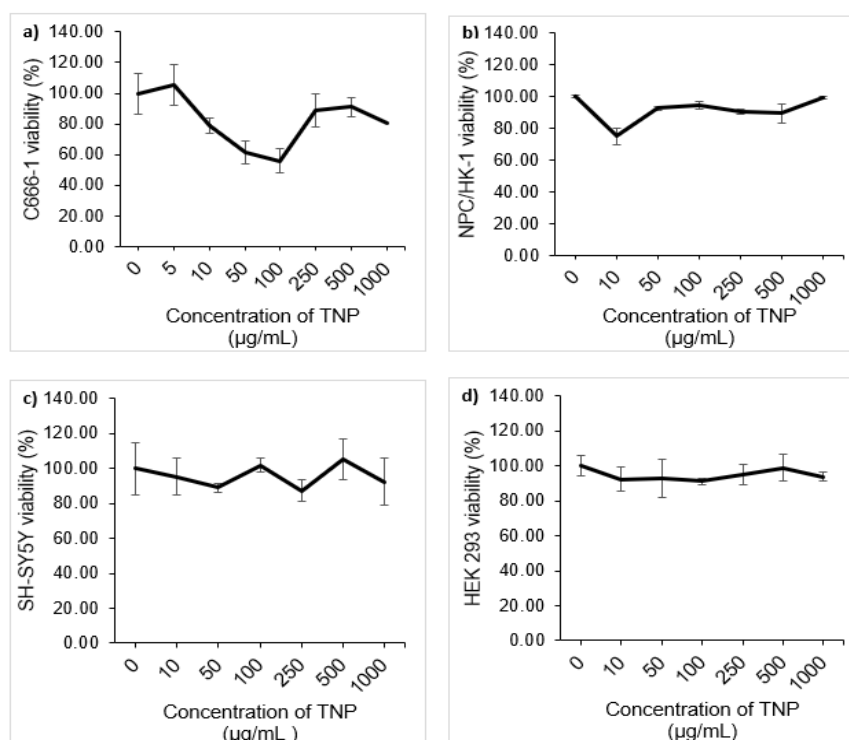


Figure 2. Cytocompatibility of TNP on different human cell lines. a) represented NPC cells; C666-1, b) NPC/HK-1, c) neuroblastoma cells; SH-SY5Y, and d) human embryonic kidney; HEK 293. Inhibition effect was observed on NPC cells with more than 25 % in inhibition after 24-h; meanwhile, the other two cells had not been affected by the TNP treatment. Statistically, no significant inhibition of all concentrations compared to untreated cells ($p = 1.000$).

3.3. Cytotoxicity of C666-1.

Similarly, the morphology of the cells observed on NPC/HK-1 also presented with the same outcome with C666-1. The cell arrangement became loose after adding CDDP compared to control (Figure 5). However, although the cell viability decreases after combination, the inhibition was insignificant ($p = 1.000$), as shown in Figure 6. Cell viability after CDDP + 100 $\mu\text{g/mL}$ treatment recorded 29.98 % meanwhile CDDP + 1000 $\mu\text{g/mL}$ showed 33.70 % viability. Those outcomes show the synergistic interaction of the treatments delivered as the percentage of cells that survive is lower than a single treatment.

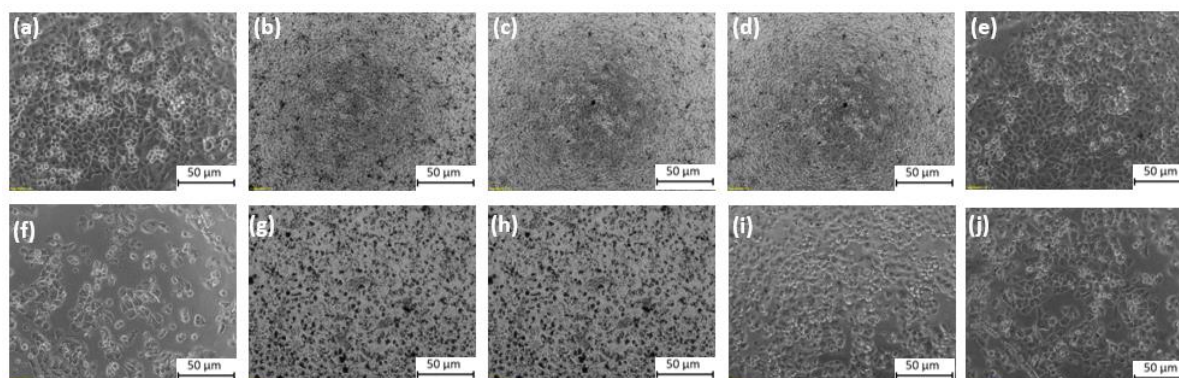


Figure 3. The microscopy analysis of C666-1 under bright field microscope after TNP exposure. Observation was compared with (a) untreated or control cell, (b) cell treated with 1000 $\mu\text{g/mL}$ TNP, (c) 100 $\mu\text{g/mL}$ TNP, (d) 50 $\mu\text{g/mL}$ TNP, (e) 25 $\mu\text{g/mL}$ TNP, (f) CDDP, (g) CDDP + 1000 $\mu\text{g/mL}$ TNP, (h) CDDP + 100 $\mu\text{g/mL}$ TNP, (i) CDDP + 50 $\mu\text{g/mL}$ TNP and (j) CDDP + 25 $\mu\text{g/mL}$ TNP. The black points on some of the images represented the presence of TNP which at higher concentration (g), the appearance was obvious compared to others.

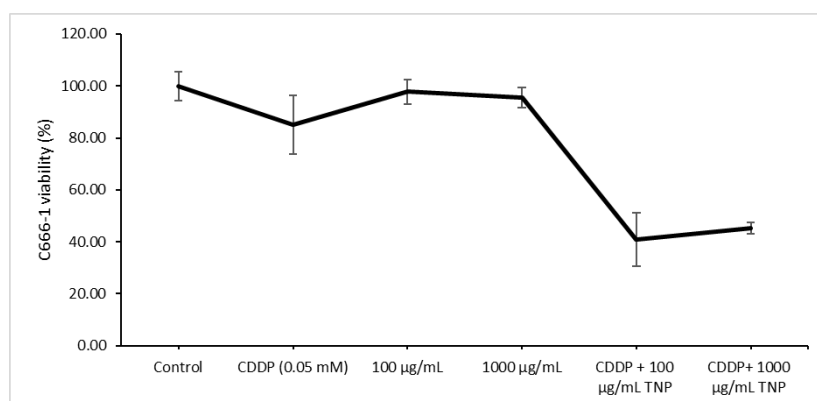


Figure 4. Cell viability of C666-1 after treatment with CDDP combined with multiple concentrations of TNP. The effect of the treatment was evaluated with MTS reagent at 495 nm after 24-h exposure. Each sample was analyzed in triplicate, normalized with control (untreated cell), and expressed in mean $\pm$ SD with no significance between each sample, $p = 1.000$.

3.4. Cytotoxicity of NPC/HK-1.

Due to its classification as a reference nanomaterial by the Organization for Economic Co-operation and Development (OECD), the commercial TNP was utilized in this study, which is also implied in the work Brandão *et al.* [17]. Although many studies had been conducted with this commercial nanomaterial, the OECD had encouraged more *in vitro* and *in vivo* work to obtain various data at different biological levels. Generally, to avoid agglomeration, the TNP usually be dispersed at high speed before being introduced to the studied culture. In a study by Vidmar *et al.* [18], ultrasonication with chemicals or ultrasonication alone was the best method to disperse the TNP before being used. The stock of the TNP was prepared according to the latter method. Filtering and centrifugation were performed to remove the undispersed TNP, resulting in a clear cell treatment solution. As displayed in Figure 1, the loss of the TNP might be explained by the result above; hence no inhibition was observed. Presence of cloudy and precipitation of cells at the highest concentration concurrently with the microscopy observation of the NPC cells as displayed in Figures 4 and 6. As demonstrated in Figure 2, upon treatment with TNP, no significant inhibition of cells was recorded, which might suggest the lower concentration of TNP might be safe to be directly applied to cells. A similar outcome of Figure 2 was shared in work by Çeşmeli & Avci [19] showed that TNP is safe up to 4 mM for radiosensitizer application. Various treatments and studies of NPC modification, especially for EBV-related NPC, have been reported. The EBV's presence had been described as one of the leading causes of NPC susceptibility, either to chemo drugs or combination therapy [20]. This work shows that C666-1 had displayed no significant inhibition even after the highest concentration was introduced compared to no-EBV-related NPC. Concurrently, Tan [21] suggested targeting EBV as an alternative for NPC treatment, demonstrating the importance of EBV in the susceptibility of NPC.

In comparison to EBV-related NPC, recurrent NPC also challenges in the NPC treatment with Pan and co-workers had agreed in targeting DNA for the efficient treatment. Recently, Liu *et al.* [22] studied the combination of TiO₂ nanotube and CDDP *in vivo* work and focused on drug delivery activity, which is the opposite of our work regarding the morphology of TiO₂ studied. Due to the difference in the purpose of the study, TNP was more favorable in targeted cancer cells by destroying the cell membrane upon attachment of p25 TNP, as explained in work by Markowska-Szczupak *et al.* [23]. CDDP had been known to damage the double strands of DNA [24]; however, direct delivery leads to toxicity; therefore,

combination with TNP was seen as another approach for future generations application which is illustrated in Figure 7. In contrast to this work, Balachandran *et al.* [25] reported the effectiveness of lung cancer treatment when the cell was treated with TNP followed by UV irradiation. More works on radiosensitizer, known as sonosensitiser, explore TNP and radiation for cancer therapy [26]. Other applications of TNP had been focusing on the uses of TNP as a radiosensitizer, which has proven to enhance the effectiveness of treatment delivered, primarily when performed on combination treatment. However, even with a different approach of TNP in NPC treatment, our work supported with Salama *et al.* [16] agreed that the combination treatment of TNP and CDDP would improve the treatment effectiveness and overcome multidrug resistance, especially when the CDDP is still the most known chemo drug for cancer therapy [27]. The potential of TNP had yet been fully discovered, especially with multiple contradicting reports of its application in the biomedical field. However, this preliminary work has shown positive interaction between TNP and CDDP in targeting NPC. Conclusion: TNP and CDDP's synergistic effect was significantly dependent on the dose of TNP employed, indicating the necessity of TNP's preliminary work. TNP's effect on cancer cells differs depending on the type of cancer cell, as seen in undifferentiated and differentiated NPC. The positive outcome decreases NPC viability, suggesting its potential in cancer therapy. More research into the intracellular interaction and the interaction between CDDP and TNP will contribute to better understanding the combination's dual effects, notably in the treatment of NPC.

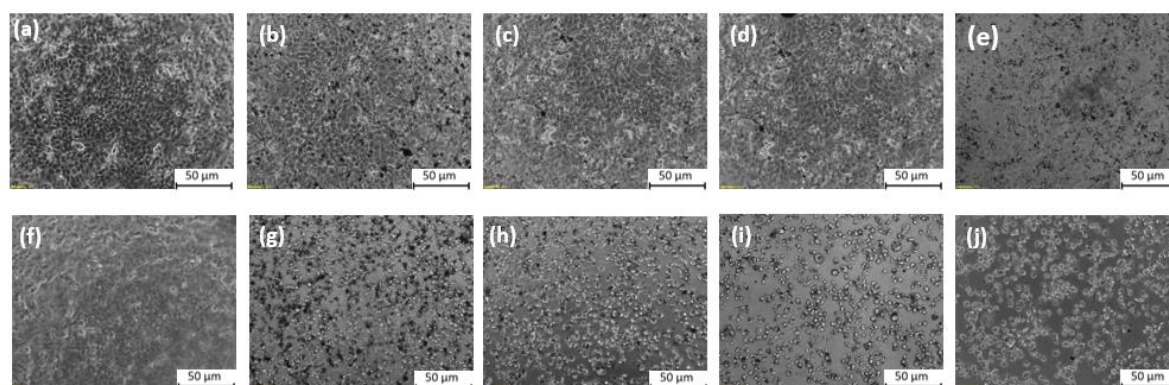


Figure 5. The microscopy analysis of NPC/HK-1 under bright field microscope. Observation was compared with (a) untreated or control cell, (b) cell treated with 1000 µg/mL TNP, (c) 100 µg/mL TNP, (d) 50 µg/mL TNP, (e) 25 µg/mL TNP, (f) CDDP, (g) CDDP + 1000 µg/mL TNP, (h) CDDP + 100 µg/mL TNP, (i) CDDP + 50 µg/mL TNP and (j) CDDP + 25 µg/mL TNP. The black points on some of the images represented the presence of TNP which at higher concentration (g), the appearance was obvious compared to others.

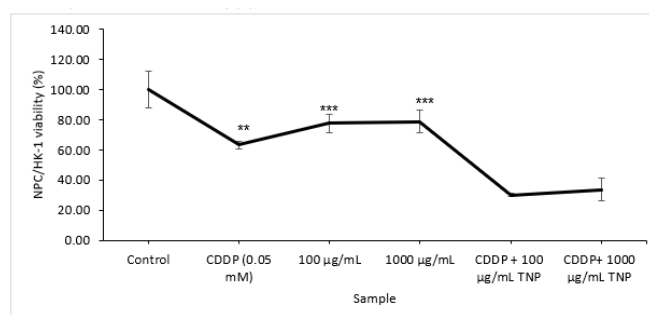


Figure 6. Cell viability of NPC/HK-1 after treatment with CDDP combined with multiple concentrations of TNP. The effect of the treatment was evaluated with MTS reagent at 495 nm after 24-h exposure. Each sample was analysed in triplicate, normalized with control (untreated cell) and expressed in mean $\pm$ SD. The significant difference observed for NPC/HK-1 ($p < 0.05$) with asterisk (**) indicated ($p = 0.01-0.001$) and (***) indicated $p < 0.001$.

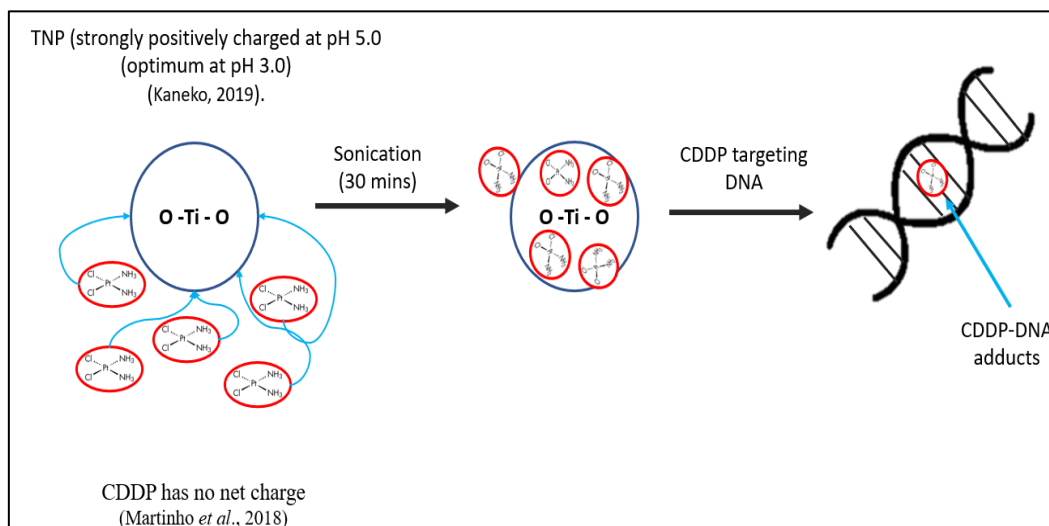


Figure 7. The interaction of TNP and CDDP on DNA damage was depicted schematically. TNP's negative charge at low pH [28] indicates that it could be used as a carrier for CDDP's with acidic properties (no net charge) [29] to enter the DNA sequence. When CDDP interacts with double-stranded DNA, it forms a DNA adduct, thus leading to cell death.

4. Conclusions

TNP and CDDP's synergistic effect was significantly dependent on the dose of TNP employed, indicating the necessity of TNP's preliminary work. TNP's effect on cancer cells differs depending on the type of cancer cell, as seen in undifferentiated and differentiated NPC. The positive outcome decreases NPC viability, suggesting its potential in cancer therapy. More research into the intracellular interaction and the interaction between CDDP and TNP will contribute to better understanding the combination's dual effects, notably in the treatment of NPC.

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Conflicts of Interest

The authors declare no conflict of interest.

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