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In Vitro Characteristics of a Combination of Thymoquinone-Resveratrol Loaded and Targeted Nanodrug Delivery System

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ABSTRACT

The objective here is to study the *in vitro* characteristics of a combination of anticancer agents made of Thymoquinone and Resveratrol (TQ-RES), when loaded into our previously prepared targeted nanodrug delivery system (TNDDS). Our system based on silica nanoparticles (NPs) and modified with a long polymer, Carboxymethyl-β-Cyclodextrin (CM-β-CD) and folic acid (FA), respectively. The Encapsulation Efficiency (EE) and the release rate were measured using UV Spectrophotometer. The loading capacity (LC) was calculated using a specific equation and Thermal Gravimetric Analysis (TGA). The cell toxicity and apoptosis induction were measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay and by determining the Caspase-3 Activity, respectively. The (EE) and the (LC) were high (88% and more than 2%, respectively). The release rate of (TQ-RES) from targeted NPs was slower than the free drugs within the first few hours, but became similar after 24 hours. The percentages of cell toxicity were also similar for these samples. However, they were higher compared to loaded-nontargeted NPs and unloaded-targeted NPs. The same trend was noticed for the percentages of cell apoptosis. Attaining the benefits of this TNDDS will open the door for further *in vivo* investigations and hence its use for targeted treatment of cancer.

Keywords: Thymoquinone; resveratrol; targeting; *in vitro* release; toxicity; apoptosis.

INTRODUCTION

Cancer is a major public health affliction in all countries. It starts when cells grow out of control and crowd out normal cells. Without treatment, tumor begins to spread throughout the body and cancer will be life threatening.[1] Cancer treatment is achieved by using one or more anticancer agents as a part of standardized chemotherapy regimen. These agents are capable of preventing or inhibiting the process of carcinogenesis by targeting multiple cancer hallmark.[1] Several plant derived compounds, such as Thymoquinone (TQ) and Resveratrol (RES), have been demonstrated to be efficient for the prevention and treatment of cancer and thus have been isolated and become important and useful anticancer agents. [1,2]

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TQ is a phytochemical compound and the predominant active component of the volatile oil extracted from black seed [*Nigella sativa* (*N. sativa*)]. [3] It possesses a variety of therapeutic effects including antioxidant, anti-inflammatory, and chemo-sensitizing. [3] More importantly, it has been tested for its efficacy as an anticancer agent. [3] RES is defined as a part of group of compounds called polyphenols and known as 3, 5, 4′ -trihydroxy-trans-stilbene which belongs to phytoalexins. [4] It has been isolated from several plants including grapes, blueberry, mulberry, peanuts and cranberry. [5] RES exhibits strong antioxidant, anti-inflammatory, anti-carcinogenesis and chemo-sensitizing effects. [6] As an anticancer agent, it has the ability to inhibit the growth of a variety of cancer cells in human, including stomach, breast, colon, prostate, thyroid and pancreatic cancers. [7] In addition, it has been investigated in a variety of other diseases including cardiovascular, diabetes, kidney and liver. [8]

Orientations have been recently shifted toward combination therapy rather than using mono therapies. [9,10] This is due to the fact that important outcomes can be achieved using combination chemotherapy which are not possible with mono-agent therapy. For instance, it provides the maximum cell toxicity within the range tolerated by the patient for each drug and it prevents or minimizes the development of new cell lines which could be drug-resistant. [10,11] A recent study, for instance, has demonstrated that TQ and RES, as free drug combination, exhibit a synergistic effect against breast cancer implanted in mice. [12] In their study, they have confirmed that RES overlaps with the same pathways triggered by TQ and thus augments its anticancer activity. Moreover, comparing the IC_{50} values of single treatments of TQ and RES, their combination resulted in a significant reduction in IC_{50} values. Other studies have evaluated the *in vitro* characteristics of each of TQ and RES when encapsulated into different nanocarriers. [13–16] To our knowledge, these characteristics have not been studied when both encapsulated, as a combination, into nanocarriers. The natural combination of (TQ-RES) is effective and relatively inexpensive, so it will be promising to further investigate its anticancer activity not only as free combination but also when encapsulated into targeted nanodrug delivery systems (TNDDSs).

TNDDSs have been embarked for treating cancer for many reasons. For instance, they can improve the solubility and bioavailability of many anticancer agents, including TQ and RES. [13] Also, they can enhance the anticancer activity of many agents by reducing their undesired and toxic effects toward normal cells through site-specific drug delivery (targeting effect). [17] We have recently demonstrated the efficacy of a novel TNDDS which was prepared by us and characterized by its high encapsulation efficiency (more than 80%) and unique surface modification. [18]

Thus, based on the results which confirmed the efficiency of (TQ-RES) as an excellent anticancer combination and on the fact that the *in vitro* characteristics of such recently investigated combinations need to be further examined, we aimed in our study to evaluate the drug release rate, cell toxicity and apoptosis induction ability of this combination when loaded into such a TNDDS.

MATERIALS AND METHODS

Materials

All of the following materials were obtained from Sigma Aldrich: FITC-labeled propylcarboxylic acid functionalized silica NPs (diameter 4 nm, particle size 200 nm), thymoquinone (99%), resveratrol (99%), carboxymethyl-β-cyclodextrin sodium salts (CM-β-CD), poly (propyleneglycol)bis(2-aminopropyletherdiamine D4000) and all reagents used for cell culture experiments. Bioworld was the source of Folic acid (purity > 98%) and Phosphate Buffered Saline (PBS, PH = 7.4).

Preparation of TQ and RES stock solutions

Stock solutions, with concentrations found within the range of previously reported values, of 53.38 μM TQ and 117 μM RES were freshly prepared in ethanol. [12] Since resveratrol is highly unstable and readily oxidizes upon contact with air and by exposure to light, [8] the preparation of stock solution was carried under dark and the glasswares were covered with aluminum foil before starting the work. For drug loading steps, ratios with 1:1 (v/v) of these drugs were used directly from the stock solutions.

Preparation of (TQ-RES) loaded silica NPs

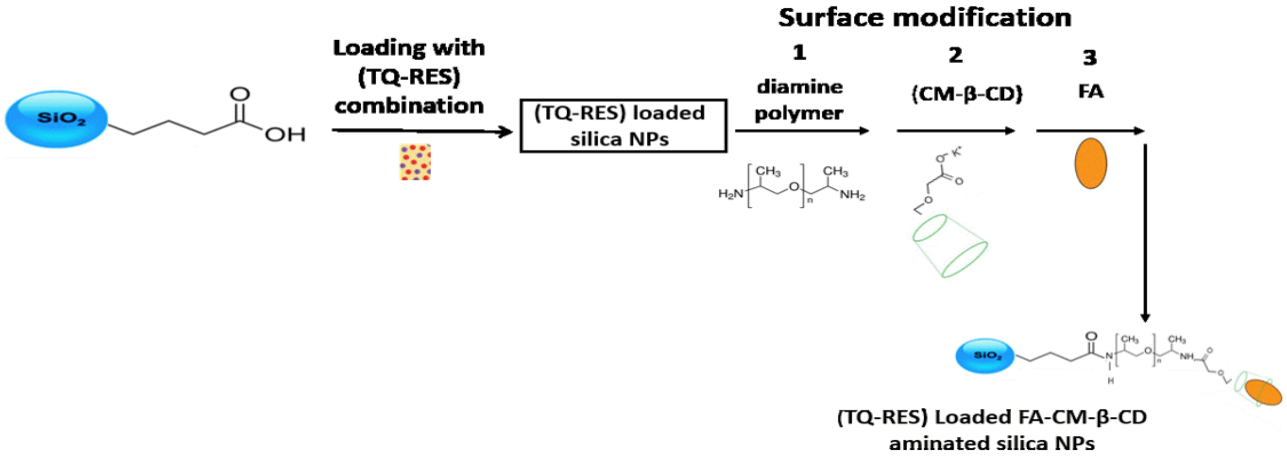
The drug loading of NPs was performed at the beginning, before their surface modification, based on our previously used method [18] . A certain amount of commercially available pure (unmodified) silica NPs (0.01g) were mixed with 2 ml of TQ-RES 1:1 (v/v) from their stock solutions, using an aluminum foil covered flask. Mixture was then stirred under dark for 24 hours. The NPs were then centrifuged at 14000 rpm for 20 minutes, washed with deionized water, centrifuged again and dried for the next day at T= 80° C.

Preparation and characterizations of FA-CM-β-CD aminated silica NPs (Surface modification)

The Procedures for synthesis of this TNDDS, which is called FA-CM-β-CD aminated silica NPs, were presented, step by step, in our previous work (scheme

1).[18] In summary, the diamine polymer (Poly (propylene glycol) bis (2-aminopropyl ether)) was first attached to the commercially available propylcarboxylic acid functionalized silica NPs, through carbodiimide coupling, to leave free amine groups on the surface. CM-β-CD was then conjugated to amine groups using the same reagent. Finally, the targeting effect was achieved by inserting FA, via host-guest interaction, into the cavity of CM-β-CD forming this system.

Using Fourier transform infrared spectroscopy (FT-IR), distinct peaks were shown directly after the conjugation of each compound.[18] A further confirmation of this successful conjugation was investigated using Dynamic Light Scattering (DLS). Its results showed that the mean particle size increased after each conjugation step.[18]Also, the Polydispersity (PD) was measured using DLS and found to be acceptable. [18]



Scheme 1. Preparation of (TQ-RES) Loaded FA-CM-β-CD aminated silica NPs.

Measuring the encapsulation efficiency of the NPs

A mixture consisting of 0.01 g of pure NPs and 2 ml of (TQ-RES) 1:1 (v/v), from their freshly prepared stock solutions, was stirred for 24 hours. The mixture was then centrifuged at 14000 rpm for 25 minutes to collect and separate the NPs and the supernatant. Another sample, prepared simultaneously and consisting only of 2 ml stock solutions of (TQ-RES) 1:1 (v/v), was also kept for 24 hours. Both samples were covered and prepared in dark. The EE was measured using the UV spectrophotometer (Uv/vis spectroscopy spuv_19) to separately measure the absorbance of both the collected supernatant and the stock solutions corresponds to both TQ (λ_{max} at 256 nm) and RES (λ_{max} at 304 nm), based on the standard calibration curves of each drug. The encapsulation efficiency (% EE) was calculated using the below equation:

(EE %) = weight of drug combination inside NPs / weight of drug combination in stock mixture……equation (1)

In the above equation, the weight of drug combination inside NPs and in stock mixture was calculated from the absorbance values as follows:

(Absorbance of drug combination in stock mixture – Absorbance of drug combination in supernatant) / Absorbance of drug combination in stock mixture).

The mean value was calculated for all the measurements which were performed in triplicate.

Measuring the loading capacity of the NPs

Loading capacity (LC %) was measured using the following reported equation: [19,20]

% LC = (weight of initial amount of drug – weight of free drug in supernatant) / weight of nanoparticles x 100……equation (2)

(LC %) was also measured using thermogravimetric analysis (TGA) (Netzsch Sta 409 PC). For this measurement, similar amounts of each of loaded and unloaded silica NPs were analyzed and their weight loss (%TG) was obtained at different temperatures (25 -1000°C). The samples were put in a TG unit using alumina pan with a rate of heating of 10 °C/minute and a maximum temperature of 1000 °C in nitrogen atmosphere.

***In vitro* drug release study**

A mixture consisting of 2 ml of PBS and 0.005 g of drug loaded and targeted NPs (FA-CM-β-CD aminated silica NPs) was put in a dialysis bag (SnakeSkin Dialysis Tubing, 22 mm _ 35 feet dry diameter). The mixture was then added to a beaker containing 6.3 ml of PBS and shaken in water path at 37° C. At different time intervals (1, 4, 5, 24, 26 and 30 hours), 1.5 ml aliquots were removed and the same volume of fresh PBS was then added. Concentration of drugs was analyzed by measuring their absorbance at 256 nm for TQ and 304 nm for RES using UV spectroscopy (Uv/vis spectroscopy spuv-19).

For free drugs measurements, accurate amounts of TQ and RES (0.0002 g and 0.0007 g, respectively) were mixed with 25 ml of PBS and shaken in water path at 37° C. The same procedure, used for the above sample, was then followed. The average was calculated for all experiments which were conducted in triplicate.

***In vitro* cell viability assay**

HeLa cells were grown at a concentration of 15000 cells/well in a (96-well) tissue culture plate in a complete tissue culture medium which consists of 10% fetal bovine serum, 1% L- glutamine, 1% penicillin streptomycin and 0.1% gentamycin solution. Next day, the media were completely removed and the adhered cells were exposed, in triplicates, to 200 µl of three different suspensions of silica NPs (0.05 mg/ml) as well as free drug combination. Plates were then incubated for two days in CO_2 incubator and the cell viability was measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay. From each well, 100 µl of medium was removed and 10 µl of thiazolyl blue tetrazolium solution was then added and incubated for 3 hours. MTT solubilization solution (100 µl/ well) was then added to stop the reactions, mixed well and incubated for another one hour. Microplate reader was used to measure the absorbance at 550 nm. Cell viability was then measured and the values were used to calculate the percentage of cell toxicity (100% - % cell viability).

Determination of Caspase-3 activity in HeLa cell line (apoptosis detection)

In the early stages of apoptosis process, the caspase-3 activity increases through a protease cascade. After treating the cells, in triplicates, with three different suspensions of silica NPs (0.05 mg/ml) as well as free drug combination, the cells were washed with ice-cold PBS and lysed using (caspase–3 assay kit, catalogue # ab39401; abcam, Missouri, USA), as a cell lysis buffer. Samples were kept in ice for 10 minutes and centrifuged at 12,000 rpm and 4 °C for 5 minutes to collect the cellular debris. The supernatant was collected and the caspase -3 activity was measured in a spectrophotometer, using DEVD-p-nitroanilide as a substrate, according to the manufacturer's instructions provided with the assay kit

RESULTS AND DISCUSSION

Measuring the encapsulation efficiency and loading capacity of NPs

To study the encapsulation efficiency (EE) of this drug combination into silica NPs, drug loading was performed at the beginning before any surface modification (Scheme 1). Using equation 1, we have found that this combination can be encapsulated in a high percentage reached to 88%.

Loading capacity (LC) of NPs was also calculated before surface modification and using equation 2, it was found to be 2.8%. LC was also determined from TGA for unmodified NPs (both unloaded and loaded) (Figures 1a and b, respectively). There was a weight loss of about 11% observed initially at 100 °C as a result of water evaporation which was adsorbed onto the unloaded NPs. The percentage of residual

silica obtained from the instrument at temperature of 1000° C was 66.31% (Figure 1a). From these results, the weight loss due to decomposition of propylcarboxylic acid group from NPs was calculated as follows:

100%- 11% - 66.31% = 22.69%

This number was very close to our previously reported value (23.14%) for unloaded and unmodified NPs when measured by TGA [18]. On the other hand, (TQ-RES) loaded NPs (Figure 1b), showed about 4% of weight loss due to water evaporation and 71.07% for the residual mass of silica. Accordingly, the weight loss due to drugs decomposition was calculated as shown below:

= 100% – 71.07% – 4% – 22.69% = 2.24%

As noticed, the obtained values of LC using equation (2) and TGA were very close and this normal difference is most likely due to differences in the amount of moisture adsorbed onto silica NPs. [21]

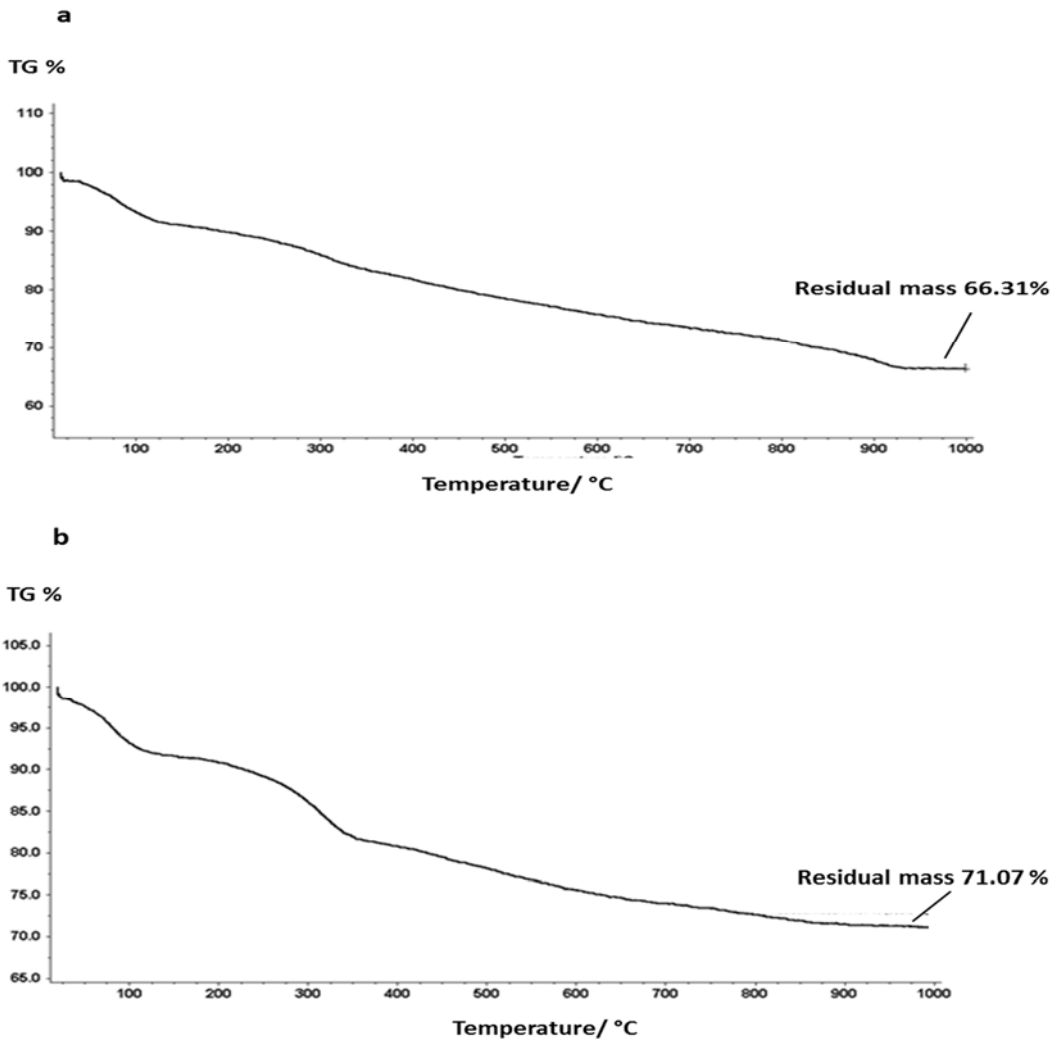


Figure 1: TGA results of (a) unloaded NPs and (b) (TQ-RES) loaded NPs.

***In vitro* drug release evaluation**

The in vitro drug release rates were studied and compared for both free (TQ-RES) and (TQ-RES) loaded and targeted NPs (FA-CM-β-CD aminated silica NPs) at physiological conditions (at pH= 7.4 and 37°C) (Figure 2). The percentages of cumulative drug release were calculated at different time intervals (1, 4, 5, 24, 26 and 30 hours). The results obtained in Figure 2 showed a faster release rate of free drug combination compared to loaded form, with a pulsatile release behavior for both, within the first five hours. However, both forms showed similar rates with a maximum value reached to 37%, after 24 hours. TQ and RES are hydrophobic drugs [22,23] and the solubility of such compounds is one of the factors that affect the drug release rate. [24] Moreover, the extent of ionization of such weak organic compounds depends mainly on the pH value. Since this study was conducted in PBS (pH= 7.4), in which these drugs are found mainly in their unionized forms, it would be reasonable to generally observe a slow rate for this combination. Even though both free and loaded forms exhibited a similar and a slow rate after 24 hours, there are many advantages can be fulfilled from using this system.

In general, TNDDSs are able to generate synergistic effects by delivering two or more drugs simultaneously through combination therapy. [25] Also, it has been reported that TNDDSs based on silica NPs improve the solubility of hydrophobic drugs. Thus, they may enhance their absorption and bioavailability [23,26,27] and minimize the use of organic solvents.

More importantly, the use of TNDDSs for the delivery of anticancer agents would be more important for systemic administration. Upon intravenous injection, the free and small molecules could not directly interact with the target cells since they diffuse nonspecifically in the body and thus lead to undesirable side effects. In contrast, the targeting achieved from TNDDS would effectively assist the anticancer agents to reach mainly to the target site and thus reduce their toxicities compared to their free form. Also, due to the fact that NPs can deliver a concentrate amount of drug in proximity to cancerous cells via targeting, [27] they will eventually increase the solubility and absorption of drug at the site of action. [28]

Moreover, small particles of less than 100 nm are susceptible to be taken by hepatocytes [29,30] which resulted in their low bioavailability and therapeutic efficacy. While in our case, the drugs are encapsulated into these 200 nm commercially available silica NPs, which would minimize this problem.

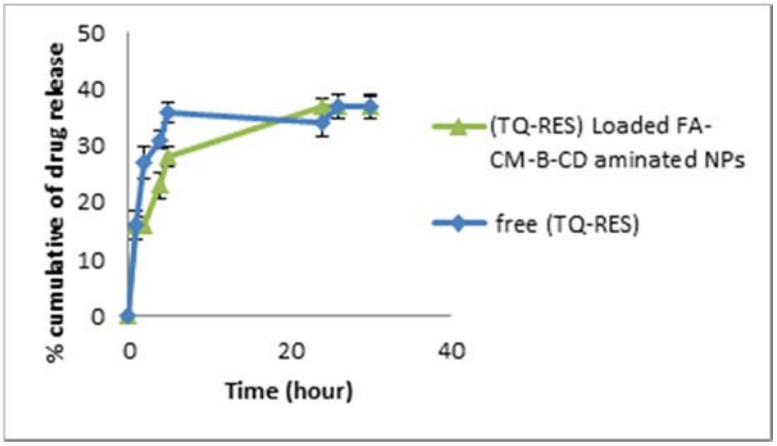


Figure 2: *In vitro* drug release rates of free and loaded (TQ-RES) at pH 7.4. Data were shown as mean ± SD (n = 3)

***In vitro* cell viability assay**

The *in vitro* cell viability assay of free combination of TQ and RES toward different cell lines was investigated by others. [12] The results confirmed that combination treatment of TQ and RES exhibited a synergism effect and significantly reduced cell viability in the tested cell lines compared to single treatment. The effect of this drug combination was further evaluated toward HeLa cell line by us. More importantly, our aim here was to investigate the cell viability of this combination when loaded into this TNDDS. We have used MTT assay which is typically used as an indicator for normal mitochondrial function and cell viability. This assay is based on revealing the activity of mitochondrial dehydrogenase which reduces MTT to blue formazan crystals.

In this part, three different suspensions of silica NPs at concentration of 0.05 mg/ml were incubated with HeLa cells for 48 hours. This particular concentration was chosen based on our previous analysis which demonstrated that concentrations of higher than 0.05 mg/ml of unmodified silica NPs leads to a significant toxicity toward HeLa cells. [18] The samples included: loaded FA-CM-β-CD aminated NPs (sample 1), which exactly represents this TNDDS, loaded CM-β-CD aminated NPs (sample 2), unloaded FA-CM-β-CD aminated NPs (sample 3) and free drug combination (sample 4). Both the drug loaded and free drug samples contained the same amount of drug combination. Sample 2, which has no FA, was prepared in order to evaluate the targeting effect achieved by this ligand. Sample 3, on the other hand, was prepared to assess the toxicity effect of this combination toward HeLa cells.

As shown in Figure 3, the toxicity effect of both loaded CM-β-CD aminated and unloaded FA-CM-β-CD aminated NPs (samples 2 and 3, respectively) were close to each other and their effects were about 40% less than that of loaded FA-CM-β-CD aminated NPs (sample 1). These expected results confirmed the advantages achieved from (TQ-RES) when encapsulated into this TNDDS. The percentage of cell toxicity of free combination (sample 4) was about 50% which is in agreement to the effect achieved from the original study of this combination toward other cancerous cell lines. [12] It was also obvious that the effect of free combination is close to sample 1.This is most likely due to the fact that drug release profile has a direct effect on the therapeutic efficacy and toxicity of NPs, both in vivo and in vitro. [31] Since samples 1 and 4 exhibited a similar pattern of release rate after 24 hours, it would be acceptable for both to show a similar toxicity effect. One point should be considered here is that these results were obtained from in vitro studies, in which the samples were exposed directly to HeLa cells. In other words, these findings deserve more future work to study the in vivo characteristics of this (TQ-RES) loaded TNDDS.

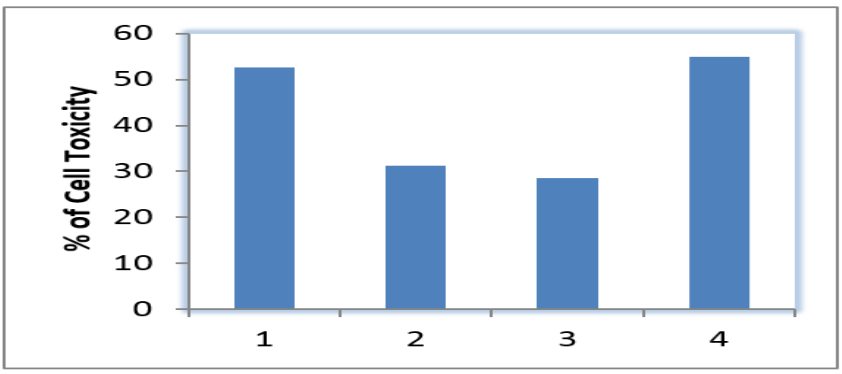


Figure 3: The percentage of cell toxicity obtained for HeLa cells when incubated for 48 hours with three different samples of silica NPs: Loaded FA-CM-β-CD aminated NPs (sample 1), loaded CM-β-CD aminated NPs (sample 2), unloaded FA-CM-β-CD aminated NPs (sample 3) as well as free drug combination (sample 4). **(SD deviation is not shown because it was almost negligible)**

***In vitro* detection of cell apoptosis**

Cell apoptosis induced by both TQ and RES has been studied *in vivo* and demonstrated that the highest degree of apoptosis obtained when a combination of TQ and RES was used in tumor bearing mice. [12] Herein, we intended to further evaluate the *in vitro* characteristics of (TQ-RES) as free combination and when loaded into this system toward HeLa cells. The same samples used in the cell viability assay were investigated here (samples 1, 2, 3 and 4). Interestingly, the order and the trend of the samples were similar to what we got from cell viability assay (Figure 4). This, indeed, further confirmed the effect obtained from each sample. Despite the fact that both free and loaded forms showed similar percentages in both cell toxicity and apoptosis induction, the advantages achieved from such TNDDSs, would encourage the use of loaded form for cancer treatment. As a summary, the percentage of cell toxicity (about 50%) obtained from these drugs as free combination was remained when encapsulated into this system. In other words, this effect was not lost and at the same time the benefits of this TNDDS can be exploited to enhance the properties of this combination.

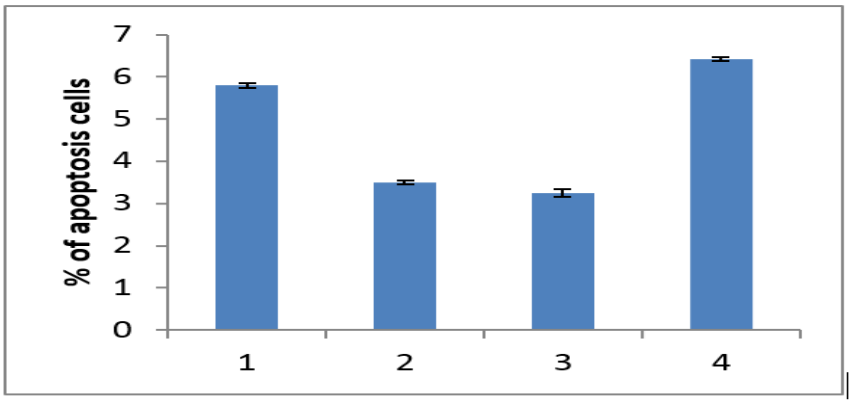


Figure 4: In vitro detection of cell apoptosis obtained for HeLa cells when incubated for 48 hours with three different samples of silica NPs: Loaded FA-CM-β-CD aminated NPs (sample 1), loaded CM-β-CD aminated NPs (sample 2), unloaded FA-CM-β-CD aminated NPs (sample 3) as well as free drug combination (sample 4). Data were shown as mean ± SD (n = 3).

CONCLUSION

In summary, our present work has examined the *in vitro* characteristics of an excellent anticancer combination made of TQ and RES when loaded into our previously developed and investigated TNDDS which is based on silica NPs. Our results confirmed a successful encapsulation of this drug combination into NPs with a high efficiency (88%) and a loading capacity of more than 2%. Release rate of (TQ-RES) from this TNNDS was slower than free drugs in the first few hours, with a pulsatile pattern for both. However, both forms showed similar rates reach to about 37%, after 24 hours. The *in vitro* cell viability and apoptosis assays were done for free drug combination and compared to targeted NPs (both loaded and unloaded) as well as drug loaded-nontargeted NPs. Both assays showed that the free drug and drug loaded into targeted NPs had similar but higher percentages compared to other samples. In spite of these similarities, this system has many advantages and would open the door for further *in vivo* investigations and hence its clinical use for targeted treatment of cancer.

AUTHOR CONTRIBUTION

Areen Khattabi conceived of the presented ideas, designed the experiments and wrote the manuscript, Eilaf

Sabbar and Wamidh Talib contributed in the experimental plan of both the cell toxicity and apoptosis assays, Diala Alqdeimat carried out the rest of the experiments.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

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الخصائص المختبرية لنظام مستهدف من الجسيمات النانونية والمحمل بمزيج من الثيموكينون والريسفيراترول

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ملخص

هدف البحث هو دراسة الخصائص المختبرية لمزيج من الأدوية المضادة للسرطان والمكونة من الثيموكينون والريسفيراترول (TQ-RES)عندما تم تحميلهم في نظام موجه لنقل الدواء الذي يعتمد على الجسيمات النانونية (nanoparticles) والذي أعددناه مسبقًا. اعتمد نظامنا هذا على استخدام جسيمات السيليكا (silica nanoparticles) المرتبطة ببوليمر و كاربوكسي ميثل β سيكلودكسترين (CM-β-CD) وحمض الفوليك (FA) على التوالي .تم حساب سعة التحميل (Encapsulation Efficiency) ومعدل الإطلاق (Release Rate) باستخدام مقياس الطيف بالأشعة فوق البنفسجية كما تم قياس كفاءة تغليف الدواء (Loading Capacity)باستخدام صيغة معينة بالاضافة الى جهاز التحليل الحراري (Thermal Gravimetric Analysis) . تم قياس سمية الخلايا واستحثاث موت الخلايا المبرمج باستخدام اختبار MTT (3 - (4 ,5- ثنائي ميثيل ثيازول – 2 – بيل) –2،5 ثنائي فينيل تيرازرازول) ومن خلال تحديد نشاطCaspase-3 ، على التوالي. كانت قيم كل من سعة التحميل والتغليف عالية (88٪ وأكثر من 2٪ على التوالي). كان معدل إطلاق ال (TQ-RES) من الجسيمات النانونية المستهدفة أبطأ من الأدوية غير المحملة خلال الخمس ساعات الأولى ولكن أصبح متشابها بعد مرور 24 ساعة. كانت نسب سمية الخلايا ايضا متشابهة لهاتين العينتين ولكن بالمقابل كانت هذه النسب أعلى مقارنة بكل من الجسيمات النانوية المحملة ولكن غير المستهدفة والجسيمات غير المحملة والمستهدفة. وقد تم ملاحظة نفس العلاقة عند دراسة نسبة استحثاث موت الخلايا المبرمج . سيؤدي الحصول على فوائد هذا النظام إلى فتح الباب للمزيد من التجارب داخل الجسم الحي وبالتالي استخدام هذا النظام كعلاج موجه للسرطان.

الكلمات الدالة: ثيموكوينون؛ ريسفيراترول؛ استهداف؛ معدل الإطلاق في المختبر؛ تسمم ؛ موت الخلايا المبرمج.

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