Clinical and Experimental Health Sciences

Synthesis of Nanoparticles Loading Indenopyrazole Derivatives and Evaluation of Biological Features

Murat Dogan[1], Umit Muhammet Kocyigit[2], Hayreddin Gezegen[3]

[1] Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Biotechnology, Sivas, Türkiye.
[2] Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Biochemistry, Sivas, Türkiye.
[3] Sivas Cumhuriyet University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Sivas, Türkiye.

Correspondence Author: Murat Dogan
E-mail: mdogan@cumhuriyet.edu.tr
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ABSTRACT

Objective: In this study, it was aimed to prepare nanoparticle formulations using chitosan, a cationic natural polymer, and tripoly phosphate, and to perform mechanical characterization and in vitro cell culture studies. In addition, the cytotoxic effects of nanoparticles containing indenopyrazol derivatives against human glioma cells (C6) and human cervical cancer cells (HeLa) were investigated.

Methods: Within the scope of the study, nanoparticles containing indenopyrazole derivative were prepared and characterization of particle size, zeta potential and morphological properties were performed. XTT cytotoxicity test was applied to evaluate the antiproliferative activities of nanoparticles containing these components.

Results: Particle size, zeta potential and morphological properties of nanoparticles were observed to be suitable for application. In vitro cell culture studies showed that nanoparticles containing indenopyrazol derivatives showed better cytotoxic effects in both cell lines.

Conclusion: The results showed that the mechanical properties of nanoparticles containing indenopyrazol derivatives are suitable and can be applied in anticancer activity studies.

Keywords: Chitosan, Indenopyrazole derivatives, Nanoparticle, Antiproliferative activity.

1. INTRODUCTION

Molecules containing indenopyrazole derivatives are compounds that show important bioactive and pharmacological activities in most studies that are widely studied in central nervous system disorders such as Alzheimer's, epilepsy and various cancer types (1). Cancer is a complex disease that can occur due to genetic and environmental factors, and its treatment can be difficult. Methods such as chemotherapy, radiation therapy, antiangiogenic drugs and immunotherapy are widely used in cancer treatment (2,3). In addition to the advantages of these methods, the problems experienced in patient compliance, the emergence of side and toxic effects reduce the treatment effectiveness of the drug (4). The undesirable effects of current treatment methods and drugs trigger the synthesis of new compounds for cancer treatment. Indenopyrazole derivatives also have effects in the treatment of cancer (5, 6). Chemically synthesized molecules can cause toxicity in different tissues or cells with the desired effect. For this reason, drug delivery systems have become a very current and important issue (7, 8). By using biocompatible polymers, targeted nanoparticular systems are synthesized and their bioactivities are investigated. In this way, the bioactivity of the synthesized chemical molecules is increased and any side effects are prevented in the application (9, 10). Chitosan, cellulose, poly vinyl alcohol, sodium alginate and many other polymers are used in the preparation of nanoparticular carrier systems (5, 11). Being biocompatible and biodegradable, showing superior release properties, polycationic structure and easy processing properties, chitosan has made it widely used in the pharmaceutical industry (12, 13). In this study, nanoparticles were prepared by ionic gelation method using chitosan and tri poly phosphate polymers, and their anticancer activities were evaluated by in vitro cell culture studies after mechanical characterization studies.

2. METHODS

2.1. Material

HeLa (ATCC CCL-2) human cervical cancer cells, and C6 (ATCC CCL-107) human glioma cells were obtained from the American Type Culture Collection (ATCC). Dulbecco's

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modified Eagle's medium (DMEM) (ATCC, Manassa, USA), fetal bovine serum (FBS) and phosphate buffer saline (PBS) were obtained from PAA Ltd. (France). L-glutamine-penicillin – streptomycin solution was purchased from Sigma-Aldrich (Steinheim am Albuch, Germany). Chitosan (medium molecular weight, 400 kDa, DD 87) were purchased from Fluka (Germany). XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) was purchased from Roche Diagnostic.

2.2. Determination of Indenopyrazole Derivatives

In this study, indenopyrazole derivatives, whose nanoparticles were prepared and characterization studies were performed, were synthesized by Gezegen et al. within the scope of TUBITAK project. Nanoparticle formulations were prepared by choosing the ones with the best bioactivity results among the synthesized derivatives, mechanical characterization studies and in vitro cell culture studies were carried out. In this study, our main aim is to evaluate the increased anticancer activities of indenopyrazole derivatives embedded in a nanoparticular carrier system.

2.3. Cell Culture Studies

Cell culture studies were performed by modifying the study of Taskin *et al*., 2020 (14). Cytotoxicity of indenopyrazole derivatives and nanoparticles containing these derivatives was measured by the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell viability assay, using HeLa and C6 cell lines. Complete mediums of HeLa and C6 cell line are the same properties. Cell lines were cultured in DMEM with 1 % L-glutamine, 10 % FBS, 100 IU/mL penicillin and 10 µg/mL streptomycin in 25 cm^2 polystyrene flasks. The flasks including cells were kept at 37°C within 5 % CO_2 humidified atmosphere and were passaged when they reached around 90 % confluence. Cells were seeded at $10x10^3$ cells/well in 96-well plates with 100 µL DMEM containing 10 % FBS, and incubated overnight. Dimethyl sulfoxide (DMSO) was used as a solvent in indenopyrazole derivatives. The compounds (2a-d) and nanoparticles including compounds were suspended with DMEM with concentration of 500 µg/ml and samples were put in the 96-well plates at determined concentrations (6, 12, 25, 50, and 100 µg/ml). In addition, the same amount of DMSO was inserted in the positive control group. The cells were incubated for 24 h. Then, the medium was removed and wells were washed with 200 µL phosphate-buffered saline (PBS). Following these periods, for determination of living cells, 100 µL of transparent (colorless) DMEM and 50 µL of XTT labelling solution were added to each well and the plates were incubated for 4 h. The absorbance values of XTT-formazan were measured using microplate (ELISA) reader at 450 nm against the control, as untreated cells. According to the results IC_{50} values of derivatives and nanoparticles with derivatives were calculated. All experiments were performed three times and 5-fluorouracil (FU) were used as standard anticancer drug.

2.4. Preparation of Nanoparticles Containing Indenopyrazole Derivatives

The indenopyrazole derivatives loaded chitosan-tripolyphosphate (TPP) nanoparticles were prepared by ionic gelation method as described (15). Briefly, chitosan solution at a concentration (0.40 % w/v) were prepared using glacial acetic acid (% 0.10 V/V) as a solvent. The chitosan dissolution process were performed via a magnetic stirrer. tripolyphosphate solution (0.40 % w/v) including compounds dropped into chitosan solution (0.40 % w/v) under predetermined stirring condition. After two hours of stirring, nanoparticles were separated by centrifugation at 10.000xg for 30 minutes. The supernatant was discarded and particles were washed with bidistilled water. This process was repeated three times. Nanoparticles were stored at +4 ºC after freeze-drying. Since the unique release properties of chitosan-TPP nanoparticles will facilitate the release of indenopyrazol derivatives, it will cause these derivatives with anticancer activity to show activity. Therefore, it was decided to prepare chitosan-TPP nanoparticles

2.5. Characterization of Nanoparticles

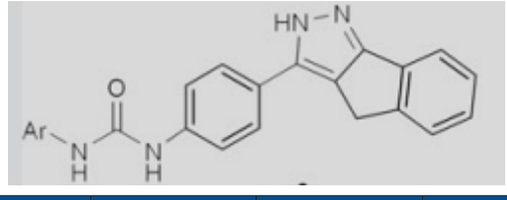
Particle size and zeta potential: Measurements of size and zeta potential of nanoparticles were performed by Zetasizer (NanoZS ZEN3500, Malvern Instrumentals Ltd., UK). The samples were suspended in phosphate buffer saline (PBS, pH 7.4) and each measurement was performed in triplicate.

Scanning electron microscope (SEM): Sample of chitosan nanoparticle was placed on metal grids with double-sided adhesive tape, coated with a gold layer using SCD 005 Sputter coater (Baltec, Liechtenstein) under 0.1 torr at room temperature. The surface morphology of nanoparticles was investigated by scanning electron microscopy (SEM; Carl Zeis-Evo 40, Germany) (16).

3. RESULTS

Indenopyrazole derivative general structure and pre-synthesized indenopyrazole derivatives were shown in Table 1.

Table 1. *Indenopyrazole derivative general structure and synthesized 2a, 2b, 2c, 2d derivatives.*



Product	Ar	Yield [%][a]	MP, °C
2a	Ph	96	355-357
2b	4-MeOPh	98	358-360
2c	4-ClPh	98	355-358
2d	3-CF3- 4-ClPh	85	357-359

Abbreviation: MP, melting point. aYield of isolated product.

3.1. Characterization of nanoparticles loading with compounds

Particle size, zeta potential and polydispersity index of nanoparticles are very important for nanoparticle entry into the cell, escape from the reticuloendothelial system and bioactivity. Therefore, these values should be in the desired ranges. The particle size and zeta potential values of the nanoparticle are in the desired range, which is very important for cell culture studies and therefore for nanoparticles to show antiproliferative activity. Results showed that zeta potential of nanoparticles ranged between 3,26± 0,05 mV and 3,78 ± 0,04 mV. The particle size of the nanoparticles between 452,32 ± 2,20 nm and 486,32 ± 2,90 nm (Table 2). Polydispersity index values of nanoparticles ranged between 0,216 ± 0,03 and 0,287 ± 0,04. The high particle size makes it difficult for the nanoparticle to pass into the cell. For this reason, nanoparticle loaded with 2c indenopyrazole derivative containing 4-chloro phenyl group have the most suitable particle size (452,32 ± 2,20 nm) for use in cell culture studies. Polydispersity index is an indicator of the homogeneity of particle sizes of nanoparticles and the difference between sizes. This value being lower than 0.4 provides an advantage in terms of application. According to the results, it was observed that the 2b indenopyrazole derivative loaded nanoparticle containing the 4-methoxy phenyl aromatic ring had the most appropriate polydispersity index value (0,216 ± 0,03). Results indicated that the nanoparticles were homogeneously dispersed in the phosphate buffer saline (PBS, pH 7.4) without forming aggregates.

Table 2. *Particle size, ζ potential, and PDI index values of nanoparticles.*

Samples	**ζ potential (mV) ± SD	Size (nm) ± SD	PDI ± SD
*NP2a	3,26 ± 0,05	486,32 ± 2,90	0,227 ± 0,05
NP2b	3,78 ± 0,04	467,56 ± 3,60	0,216 ± 0,03
NP2c	3,74 ± 0,02	452,32 ± 2,20	0,232 ± 0,02
NP2d	3,38 ± 0,04	458,44 ± 2,74	0,287 ± 0,04

**NP indicates nanoparticle, **ζ indicates zeta. SD indicates standart deviation.*

3.2. SEM image result of nanoparticles

The morphological properties of the nanoparticles were shown in Figure 1. The morphology of chitosan nanoparticles shows an almost smooth structure.

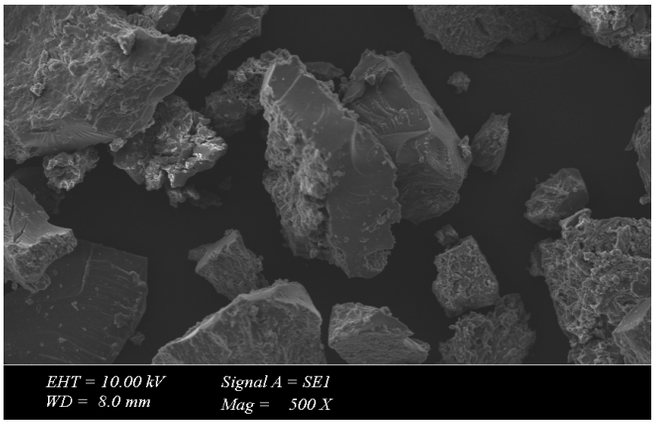


Figure 1. *SEM image of chitosan nanoparticles*

3.3. Evaluation of cytotoxic activity results of nanoparticles and indenopyrazole derivatives

Based on the results of the studies of Gezegen et al. on indenopyrazole derivatives, it was observed that these derivatives have significant efficacy in glioma and cervical cell lines (17). Therefore, in this study, it was decided to study these cells. The cytotoxic activities of 2a-d and 2a-d nanoparticles were evaluated on HeLa and C6 cell lines at 24 hours and 5-Fluorouracil (5-FU) was used as positive control. It was seen clearly from the table that all indenopyrazole compounds 2a-d and nanoparticles containing indenopyrazole compounds 2a-d against the HeLa cell line have better anticancer activity than 5-FU (Table 3). In addition, these derivatives and nanoparticles loaded with these derivatives did not show same effective cytotoxic activity on C6 cell line. The IC_{50} values of 2a-d indenopyrazole derivatives are between 7,06 ± 0,15 µg/mL and 8,09 ± 0,12µg/mL on HeLa cell line. The highest cytotoxic activity was shown by 2d with a 7,06 ± 0,15 µg/mL IC_{50} value on HeLa cells. This derivative contains 3 – trifluorine metil 4-chlorine as a substituent. According to the results, nanoparticles containing indenopyrazole derivatives show more effective cytotoxic activity than samples containing only these derivatives on HeLa cell line. Except for the 2b, both indenopyrazole derivatives and nanoparticles showed significantly better cytotoxic activity than 5-FU on HeLa cell line.

Table 3. *IC50 values of 2a-2d and NPs on HeLa and C6 cell lines (mean ± SD, n = 3).*

Samples	Ar[a]	HeLa IC_{50} (µg/mL)	C6 IC_{50} (µg/mL)
2a	Ph	7,76 ± 0,16	11,96 ± 0,32
NP2a	Ph	7,02 ± 0,21	10,12 ± 0,22
2b	4-MeOPh	8,09 ± 0,12	12,84 ± 0,16
NP2b	4-MeOPh	7,38 ± 0,18	11,05 ± 0,09
2c	4-ClPh	7,32 ± 0,31	11,68 ± 0,17
NP2c	4-ClPh	7,08 ± 0,28	10,98 ± 0,13
2d	3-CF3- 4-ClPh	7,06 ± 0,15	13,65 ± 0,23
NP2d	3-CF3- 4-ClPh	6,64 ± 0,18	11,83 ± 0,16
5-FU	5-FU	8,22 ± 0,24	7,96 ± 0,09

Abbreviations: 5-FU, 5-fluorouracil; NP, nanoparticle, SD, standard deviation. [a]For the formula, see Table 1.

However, indenopyrazole derivatives loaded nanoparticles and only derivatives did not show a more significant cytotoxic effect in the C6 cell line compared to 5-FU. 2c (IC_{50}: 11,68 ± 0,17 µg/mL) and 2d (IC_{50}:13,65 ± 0,23 µg/mL) showed the highest and lowest cytotoxic activity in C6 cell line, respectively. The IC_{50} value of 5-FU was calculated as 7,96 ± 0,09 µg/mL. The IC_{50} values of nanoparticles in the C6 cell line are between 10,12 ± 0,22 µg/mL and 11,83 ± 0,16 µg/mL. The 2a indenopyrazole compound loaded nanoparticle containing the phenyl substituent showed the highest cytotoxic effect. The results we obtained confirm our hypothesis of creating a more effective anticancer activity on cancer cells in in vitro cell culture studies, which is the aim of this study. According to the results, it was observed that nanoparticles showed

greater cytotoxic activity than indenopyrazole derivatives in both HeLa and C6 cell lines.

4. DISCUSSION

Cell culture studies of indenopyrazol derivatives and nanoparticles were carried out in HeLa and C6 cell lines. The fact that the nanoparticles were not in the desired sizes or could be aggregated could have been a limitation of this study. However, according to the results, such a problem was not observed. In order to determine the applicability of nanoparticles, particle size, zeta potential, Polydisperse index value and morphological properties should be at desired values. In order to evaluate these properties, necessary studies were performed and it was determined that the nanoparticles were suitable. Chitosan nanoparticles have enhanced encapsulation and release properties. Thanks to these features, high efficiency is achieved in the application and activity of the active substance they contain. In the light of this information, in our study, chitosan nanoparticles showed a high antiproliferative effect in C6 and HeLa cell lines in in vitro cell culture studies. Indenopyrazol derivatives show different anticancer activity depending on the functional groups in their structures. The indenopyrazole derivatives we evaluated in our study have phenyl, methoxy phenyl, chlorophenyl, trifluorochlorophenyl aromatic functional groups, respectively. According to the IC_{50} values of these derivatives, 2d containing trifluoro chlorophenyl group showed the highest antiproliferative activity, while 2b containing methoxy phenyl group showed the lowest cytotoxic activity in HeLa cell line. According to the results, it can be said that chlorine and fluorine elements attached to the phenyl ring play an important role in increasing anticancer activity in HeLa cell line. When the IC_{50} data of indenopyrazol derivatives in the C6 cell line were evaluated, it was observed that 2c and 2d showed the best and least antiproliferative activity, respectively. It was observed that the efficacy of both indenopyrazole derivatives and nanoparticles on the C6 cell line was lower than that of 5-FU. When the data in the study of Gezegen et al. were evaluated, no significant difference was observed between the IC_{50} values of indenopyrazole derivatives (17). However, it was observed that the IC_{50} values of nanoparticles containing the same amount of indenopyrazole derivatives were significantly lower than the IC_{50} values of indenopyrazole derivatives. It was observed that the preparation and application of nanoparticle formulation increased the antiproliferative activity in C6 and HeLa cell lines, and this result is very meaningful and valuable for the study.

5. CONCLUSION

According to the results of characterization and in vitro cell culture studies of nanoparticles, it was observed that nanoparticles containing indenopyrazole derivative showed antiproliferative effects in HeLa and C6 cells. In addition, it was determined that nanoparticles have higher anticancer activity than only indenopyrazol derivatives. According to the characterization results of the nanoparticles, it was shown that the particle size, zeta potential, polydisperse index and morphological properties were suitable.

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Conflict of interests

The author declare that they have no conflict of interest.

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