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Anticancer Effects of ZnO/CNT@Fe_3O_4 in AML-Derived KG1 Cells: Shedding Light on Promising Potential of Metal Nanoparticles in Acute Leukemia

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ABSTRACT
Background: Therapeutic approaches for acute myeloid leukemia (AML) have remained largely unchanged for over 40 years and cytarabine and an anthracycline (e.g., daunorubicin) backbone is the main induction therapy for these patients. Resistance to chemotherapy is the major clinical challenge and contributes to short-term survival with a high rate of disease recurrence. Given the established efficacy of nanoparticles in cancer treatment, this study was designed to evaluate the anticancer property of our novel nanocomposite in the AML-derived KG1 cells.
Materials and Methods: To assess the anti-leukemic effects of our nanocomposite on AML cells, we used MTT and trypan blue assays. Flow cytometric analysis and q-RT-PCR were also applied to evaluate the impact of nanocomposite on cell cycle and apoptosis.
Results: Our results outlined that ZnO/CNT@Fe_3O_4 decreased viability and metabolic activity of KG1 cells through induction of G1 arrest by increasing the expression of p21 and p27 cyclin-dependent kinase inhibitors and decreasing c-Myc transcription. Moreover, ZnO/CNT@Fe_3O_4 markedly elevated the percentage of apoptotic cells which was coupled with a significant alteration of Bax and Bcl-2 expressions. Synergistic experiments showed that ZnO/CNT@Fe_3O_4 enhances the cytotoxic effects of Vincristine on KG1 cells.
Conclusion: In conclusion, this study sheds light on the potent anti-leukemic effects of ZnO/CNT@Fe_3O_4 and provides evidence for the application of this agent in the treatment of acute myeloid leukemia.

Keywords: Acute myeloid leukemia (AML); Zinc oxide; Carbon nanotubes; Iron oxide; Nanoparticles; Vincristine

INTRODUCTION

Treatment of cancer has become more aggressive during the current years and severe side effects have called into question the recent advances in this field[1]. As one of the most prevalent leukemia in older adults and the fifth most common malignancy in children, acute myeloid leukemia (AML) affects about 21,450 people in the United States and resulted in 10,920 deaths in 2019 [2,3]. The present statue of therapeutic approaches for this malignancy has demonstrated low levels of hope at the end of treatment and a great number of AML patients will relapse after initial promising response[4]. Moreover, severe side effects of conventional chemotherapy highlights the necessity of novel compounds that more effectively kill the tumor cells while exhibiting the least toxicity towards normal tissues[5].

The application of nanomaterials is one of the most hopeful approaches to provide a promising solution to meet these challenges[6]. Nowadays, many publications have evaluated the application of different types of metal nanoparticles in a wide range of biomedical and medical areas. ZnO quantum dots (QDs) and Fe_3O_4 nanoparticles (NPs) were the most promising nanomaterials in which their anti-cancer effects have been proved in a broad spectrum of tumors [7, 8]. It has been reported that ZnO NPs could decrease the viability of CAL-27 [9], U87 [10] and MCF-7 [11] cell lines. Yuexia Xie et al. indicated that Fe_3O_4 NPs induces significant cytotoxic effects in two kinds of human hepatoma cell lines, SK-Hep-1 and Hep3B, mainly through mitochondria-dependent ROS generation[12]. Tanino et al. reported that ZnO NPs exerts significant cytotoxicity against lung cancer cells while they exhibited less cytotoxic effects on normal lung-derived cells and did not elicit observable adverse effects after intravenous administration[13]. The results of our recent study have demonstrated that ZnO/CNT@Fe_3O_4 significantly reduced the survival of CML-derived K562 cells through ROS generation and alteration of apoptosis-related genes [14]. The prominent efficacy of NPs against chronic myeloid leukemia cells and lack of an efficient treatment in acute leukemia encouraged us to investigate whether ZnO/CNT@Fe_3O_4 induces favorable cytotoxicity in AML cells. To this end, KG1 cells were subjected to various concentrations of ZnO/CNT@Fe_3O_4 and then cell viability index, metabolic activity, growth kinetics and transcriptional alteration of apoptosis- and cell cycle-related genes were investigated to dissect the underlying mechanisms by which ZnO/CNT@Fe_3O_4 exert its cytotoxic effect.

Materials and Methods

Synthesis and formulation of nanocomposite

Briefly, 0.5-2 gr ZnO nanoparticles (coated with hydrophilic CNT@fatty) and iron oxide NPs were used to modify 1-3 gr multiwall CNT (8-15 nm) in 20 ml pure ethanol. As synergic material, 1-2 gr zinc oxide NPs powder was added to nanofluid and the components were mixed at room temperature. Afterward, 1-2 gr PEG 6000 as a binding agent was added and the mixture was stirred at 40-50 °C for 1-2 h. Then, 2-3 ml Span 80 as a stabilizer, emulsifier, and nonionic surfactant was added with persistent vigorous stirring, under reflux processing in about 2 h duration of the reaction.

Cell lines and drug treatments

To investigate the impact of ZnO/CNT@Fe_3O_4 on acute myeloid leukemia, KG-1 cells were grown in suspension in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. The cell cultures were all kept at 37 ºC in a humidified atmosphere of 5% CO_2. The leukemic cells were then treated with the relevant amounts of ZnO/CNT@Fe_3O_4, Vincristine (VCR) (Sina Daroo, Iran), and the combination of both agents. KG1 cells were also exposed to equal amounts of solvents, as an alternative control at the final concentration of 0.1%.

Trypan blue exclusion assay

Trypan blue assay was tested on 150×10^3 leukemic cells seeded in 24-well plate in the medium containing different concentrations of ZnO/CNT@Fe_3O_4, either as a single agent or in combination with VCR. Following the indicated time intervals, centrifuged cell pellets were re-suspended in serum-free complete medium and then an equivalent amount of 0.4% trypan blue was added to each sample. The numbers of viable cells were counted manually under a light microscope and the percentage of viable cells was assessed.

Detection of metabolic activity by microculture tetrazolium test

Acute myeloid leukemia cells (5×10^3) were seeded in 96-well plate in the presence of ZnO/CNT@Fe_3O_4, as a single agent or in combined-modality with VCR, and incubated in a humidified 5% CO_2 incubator at 37 °C. The experiment process has been described in our previous article [15].

Determination of combination index (CI) and dose reduction index (DRI)

To investigate the efficacy of agents combinations, the reduction of cell survival was examined and the combination index (CI) and dose reduction index (DRI) were evaluated as described previously [16]. The CI values of <1, =1, and >1 indicate synergism, additive effect, and antagonism of agents, respectively.

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA from AML-derived KG1 cells was extracted using the RNA Isolation Kit (Roche, Mannheim, Germany). After confirming the quantity of the extracted RNA by Nanodrop instrument, the reverse transcription reaction was performed using a cDNA synthesis kit (Takara Bio, Shiga, Japan). Next, to examine the effect of ZnO/CNT@Fe3O4/VCR on the expression of proliferation- and apoptotic-related genes, cDNAs were subjected to quantitative real-time PCR (qRT-PCR). The fold change values were calculated based on the $2^{-\Delta\Delta Ct}$ relative expression formula.

Assessment of cell distribution in the cell cycle using flow cytometry

The impact of ZnO/CNT@Fe_3O_4-plus-VCR on the cell cycle progression was evaluated by PI staining. After the treatment of KG1 cells with the designated concentrations of agents, either alone or in combination, the cells were harvested, washed with cold PBS, and then fixed in 70% ethanol overnight. Afterward, for DNA staining and RNA degradation, we added PI and RNase, respectively. Cells were then incubated for a further 30 min and the distribution of cells was evaluated by flow cytometry. For interpreting the obtained data, we used the Windows FlowJo V10 software.

Detection of apoptosis using flow cytometry

To investigate the effect of ZnO/CNT@Fe_3O_4 on the induction of programmed cell death, AML cells were subjected to apoptosis analysis. Briefly, after 24h of treatment with the designated concentrations of ZnO/CNT@Fe_3O_4 in the presence or absence of VCR, KG1 cells were harvested, washed with PBS, and re-suspended in a total volume of 100 µl of the incubation buffer. After that, Annexin-V-Flous (2 µl per sample) was added, and cell suspensions were incubated for 20 min in the dark and then fluorescence was quantified using flow cytometry. Annexin V-positive and PI-negative cells were considered to be in the early apoptotic phase and cells having positive staining both for Annexin-V and PI were considered to undergo late apoptosis or necrosis.

Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.) of three independent experiments. All presented data were analyzed using GraphPad Prism Software using two-tailed student's test and one-way variance analysis. In order to compare between the control group and the drugs-treated ones, the Dennett's multiple comparison test was used. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS

Characterization techniques of the synthesized ZnO/CNT@Fe_3O_4 nanocomposite product

ZnO/CNT@Fe_3O_4 nanocomposite has been synthesized and its formulation and structure were examined by various characterization methods such as FTIR analysis, X-ray powder diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). As presented in Figure 1, FTIR Spectrum of ZnO/CNT@Fe_3O_4 indicated a high-intensity broadband at 3402 which is related to vibration mode of the hydroxyl group in water and carboxylic acid groups belonging to fatty acid in nanoproduct [17]. Symmetrical stretching vibration in 838 cm^{-1} and 721 cm^{-1} is associated with the presence of Zn-O and Fe-O in nanoformulation. Two low-intensity peaks observed at 1462 cm^{-1} and 1399 cm^{-1} are related to symmetric and asymmetric -CH3 groups respectively, which confirm the presence of polyethylene glycol (PEG) in the system [18, 19]. At least two absorption picks correlated to CNT were observed at 1110 cm^{-1}, 1735 cm^{-1} and 1645 cm^{-1} that correspond to C-O-C [20, 21], C = O [22, 23] and C = C, respectively[24]. X-ray diffraction peaks were in agreement with the results of the FTIR spectrum, where the standard XRD patterns of zinc oxide (JCPDS no.: 017-751377) and iron oxide magnetic nanoparticles (JCPDS no: 00-003-0863) confirmed the presence of aforementioned nanoparticles in the system. Moreover, the detected peak located at 2θ = 24.5 could be indexed as (002) planes of CNT[25]. FESEM micrographs confirmed the formation of nanocomposite and revealed that there are individual particles on the surface of carbon nanotubes. Consistently, transmission electron microscopy (TEM) illustrated nanostructures composed mainly of nanorods, besides few nano granules with the diameters ranged from 5 to 80 nm.

Inhibitory effects of ZnO/CNT@Fe_3O_4 on viability and metabolic activity of KG1 cells

Chemotherapy is not curative most of the time and has various complications, such as high toxicity and low specificity, emphasizing the necessity of incorporation of new therapeutic agents in AML [26]. In an effort to evaluate the effects of the newly synthesized nanocomposite on acute leukemia, KG1 cells were treated with the designated concentrations of ZnO/CNT@Fe_3O_4 and trypan blue exclusive assay and MTT assays were applied to evaluate the viability and metabolic activity, respectively. The results of time and concentration-dependent experiments demonstrated that both viability and metabolic activity were declined upon exposure of KG1 cells to ZnO/CNT@Fe_3O_4. As demonstrated in Figure 2, while ZnO/CNT@Fe_3O_4 at low concentrations excreted a minimal effect on KG1 cells, maximal repression was observed at 20 µg/ml where viability and metabolic activity decreased to 57% and 46%, respectively.

ZnO/CNT@Fe_3O_4 disrupts Bcl-2 and Bax transcription ratio in favor of apoptosis

A growing body of evidence demonstrated that metal nanoparticles decrease the survival of cancer cells through induction of apoptosis[27]. As a result, it was of great attention to assessing whether the antileukemic properties of ZnO/CNT@Fe_3O_4 in KG1 cells were because of the induction of apoptosis. The apoptotic effect of the compound against KG1 cells was confirmed by Annexin-PI stating, as we found a vigorous increase in both Annexin- and Annexin/PI-positive cells. To shed light on ZnO/CNT@Fe_3O_4-induced apoptosis at the molecular level, we analyzed the mRNA levels of Bcl-2 and Bax. As indicated in Figure 3, results of qRT-PCR demonstrated that not only ZnO/CNT@Fe_3O_4 repressed the expression of Bcl2 also exerted a robust increase in mRNA level of Bax, indicating that ZnO/CNT@Fe_3O_4 induces its apoptotic effect through disturbing the balanced ratio of Bax and Bcl2 genes.

ZnO/CNT@Fe_3O_4 induced its suppressive effects through ceasing KG1 cells in the G1 phase of the cell cycle

Based on the considerable anti-proliferative effects of ZnO/CNT@Fe_3O_4 on KG1 cells which was proved by a substantial reduction in the number of NP-treated KG1 cells, it was interesting to determine whether this growth suppressive effect was coupled with alteration in the cell cycle. As presented in Figure 4, the effect of ZnO/CNT@Fe_3O_4 on cell cycle distribution was investigated using PI staining, where we found that ZnO/CNT@Fe_3O_4 augmented the fraction of hypodiploid cells in the sub-G1 phase, which is a representative of DNA fragmentation-mediated apoptosis in KG1 cells. We also found a noticeable rise in the percentage of the cells in G1 in a concentration-dependent manner. Furthermore, DNA content analysis showed that the population of ZnO/CNT@Fe3O4-treated cells was significantly diminished in the S phase of the cell cycle. Investigating the molecular mechanisms responsible for this effect demonstrated a robust increase in the mRNA expression levels of both p21 and p27, which can impede cell cycle progression through inhibition of CDKs. In congruence, the expression of c-Myc proto-oncogene which induces positive cell-cycle regulators was remarkably decreased in a concentration-dependent manner.

the interaction between ZnO/CNT@Fe_3O_4 and VCR as one of the most common chemotherapeutic agents used in AML. While VCR at the concentration of 2 nM had slight effects on KG1 cells, its combination with ZnO/CNT@Fe_3O_4 notably decreased the viability and metabolic activity. The results of isobologram analysis and combination index (CI) showed that ZnO/CNT@Fe_3O_4 at the concentrations of 15 µg/ml and 20 µg/ml in combination with VCR synergistically decreased cell viability. As presented in Figure 5, all the points are below the line of additive effects showing a

Combination of ZnO/CNT@Fe_3O_4 and VCR reduced the survival of KG1 cells synergistically

Given the potent antileukemic effects of our synthesized nanoparticle, it was enticing to evaluate synergistic effect between these agents. Our results were also confirmed by the fraction-affect (FA) versus combination index (CI) plot that exhibits a synergistic cytotoxic effect (CI < 1) in the combination treatment. Stimulatory effects of ZnO/CNT@Fe_3O_4 on VCR cytotoxicity were further confirmed by apoptosis analysis, where we found that the percentage of both Annexin and Annexin/PI positive cells were significantly higher in combinatorial series, indicating that ZnO/CNT@Fe_3O_4 elevated the cytotoxicity of VCR through induction of apoptosis.

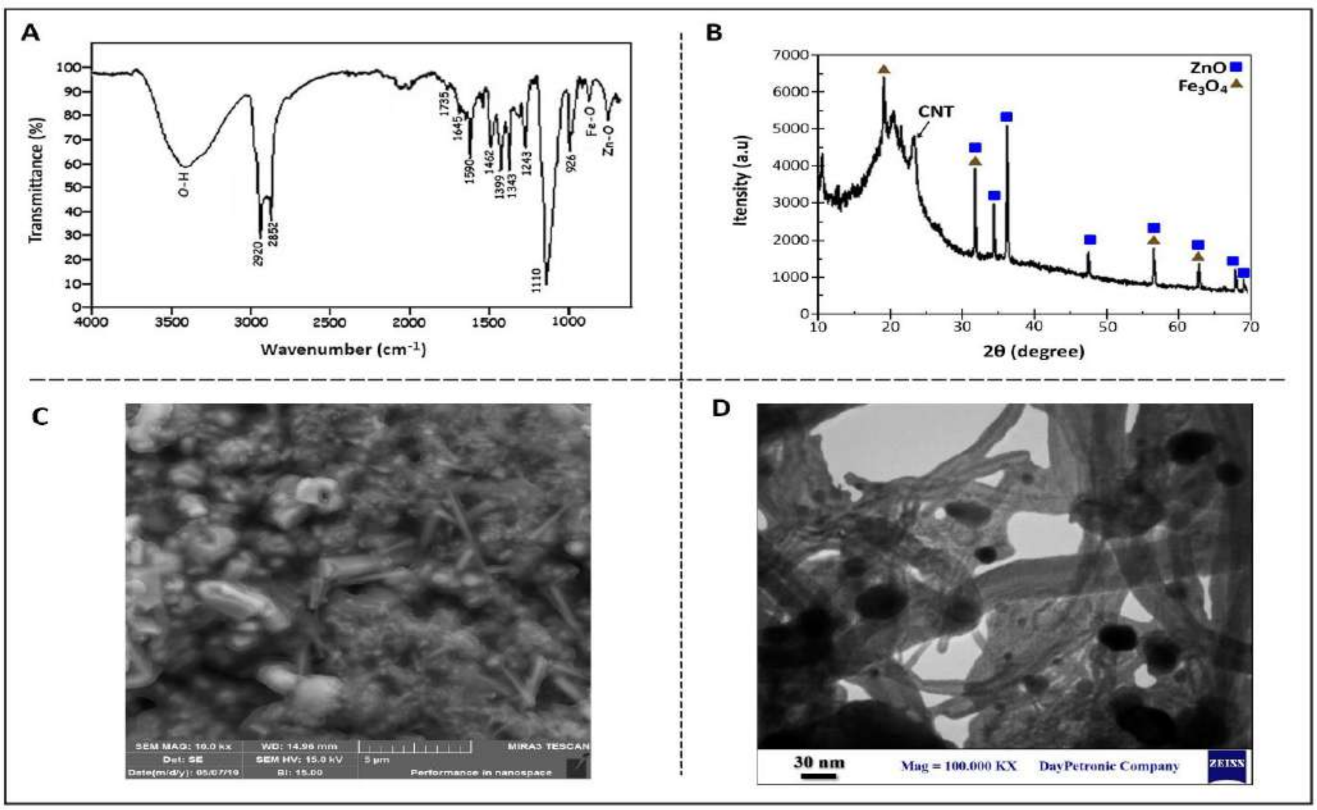

Figure 1 **A)** FTIR spectrum of ZnO/CNT@Fe_3O_4 demonstrated characteristic peaks of Zn–O and Fe–O bonds at 721 and 834 cm^{-1}, respectively. **B)** XRD analysis of ZnO/CNT@Fe_3O_4 confirmed the structural characteristics of ZnO/CNT@Fe_3O_4. **C)** Attachment of round shaped Q-dot ZnO and Fe_3O_4 NPs to CNTs was confirmed by scanning electron microscopy (SEM). **D)** Transmission electron microscopy (TEM) showed the morphology of the ZnO/CNT@Fe_3O_4 nanocomposites and the maximum size of nanoparticles was determined by about 80 nm.

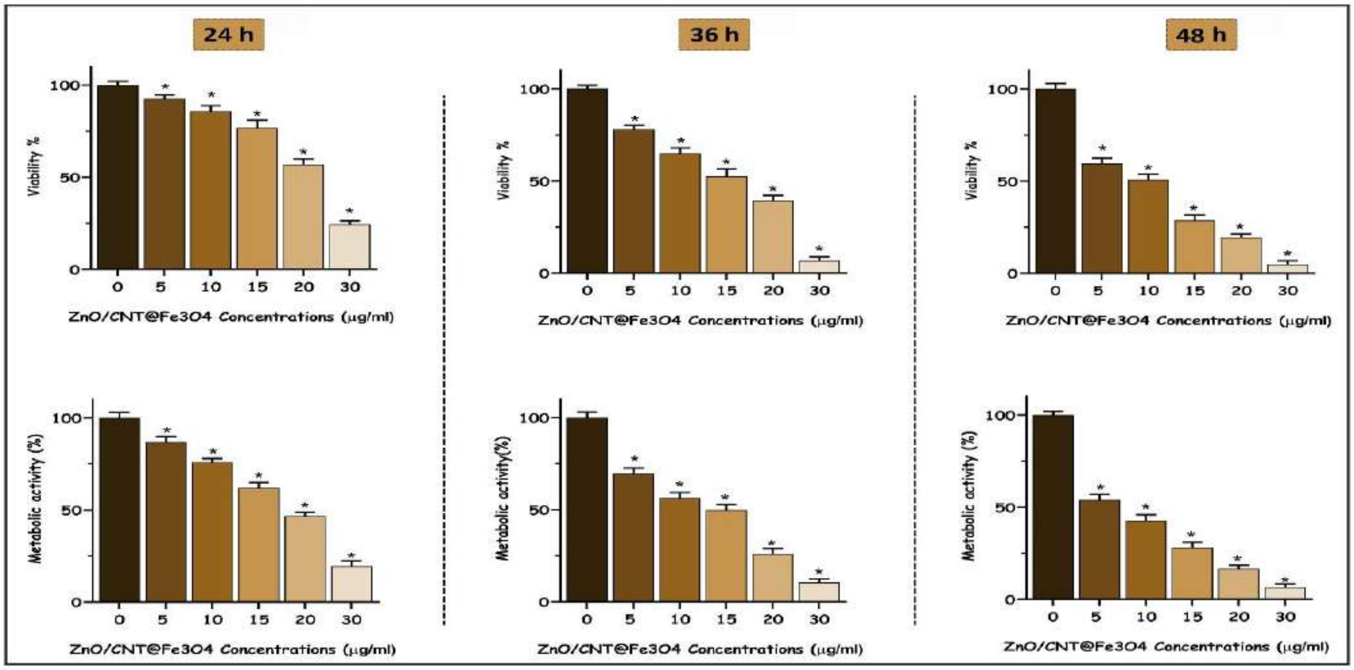

Figure 2. ZnO/CNT@Fe_3O_4 decreased both viability and metabolic activity of KG1 cells. KG1 cells were treated with nanocomposite for 24 h, 36 h, and 48 h and cell viability and metabolic activity were evaluated by trypan blue exclusion and MTT assays, respectively. As presented, ZnO/CNT@Fe_3O_4 reduced the survival of KG1 cells in concentration- and time-dependent manners. Values are given as mean ± S.D. of three independent experiments. *$P \leq 0.05$ represented significant changes from the control.

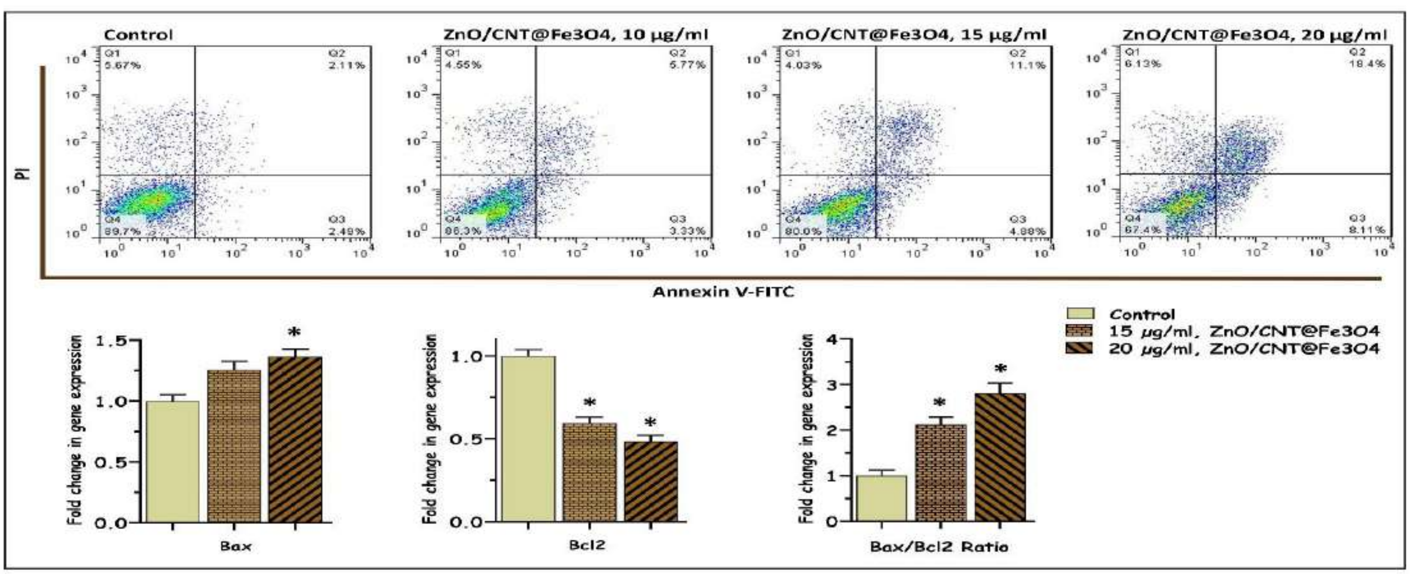

Figure 3. The apoptotic effects of ZnO/CNT@Fe_3O_4 on KG1 cells. ZnO/CNT@Fe_3O_4 increased the percentage of Annexin-V positive cells. The results of the qRT-PCR analysis revealed that ZnO/CNT@Fe_3O_4 induced apoptosis, at least partly, through the alteration of the Bax/Bcl2 ratio. Values are given as mean ± standard deviation of three independent experiments. * $P \leq 0.05$ represents significant changes from untreated control.

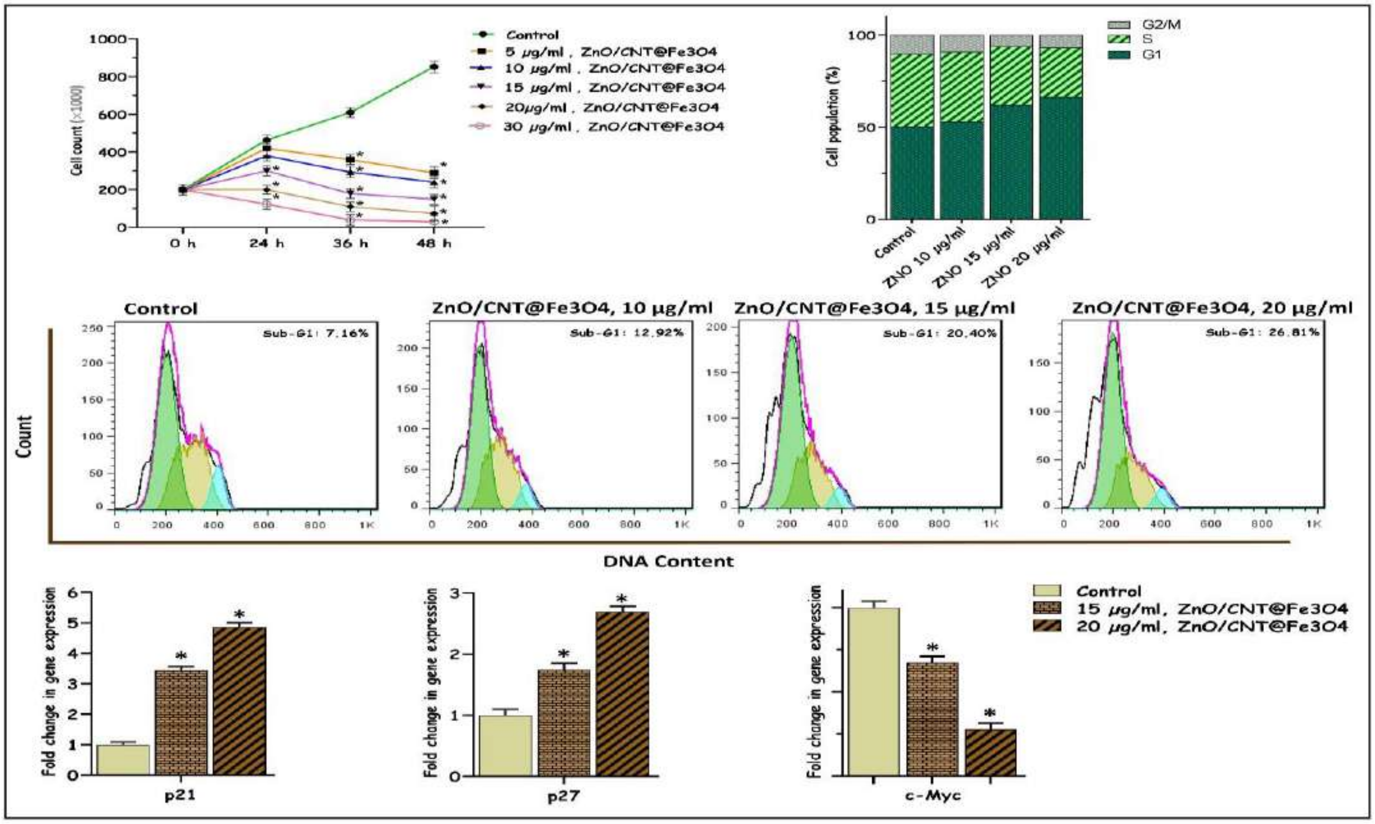

Figure 4. Our results revealed that ZnO/CNT@Fe_3O_4 diminished the proliferative capacity of the cells, as evidenced by the decreased number of KG1 cells, via the induction of G1 cell cycle arrest. While the mRNA expression levels of p21 and p27 were increased upon exposure to the agent in a concentration-dependent manner, c-Myc transcription was decreased significantly. Values are given as mean ± S.D. of three independent experiments. *$P \leq 0.05$ represented significant changes from the control.

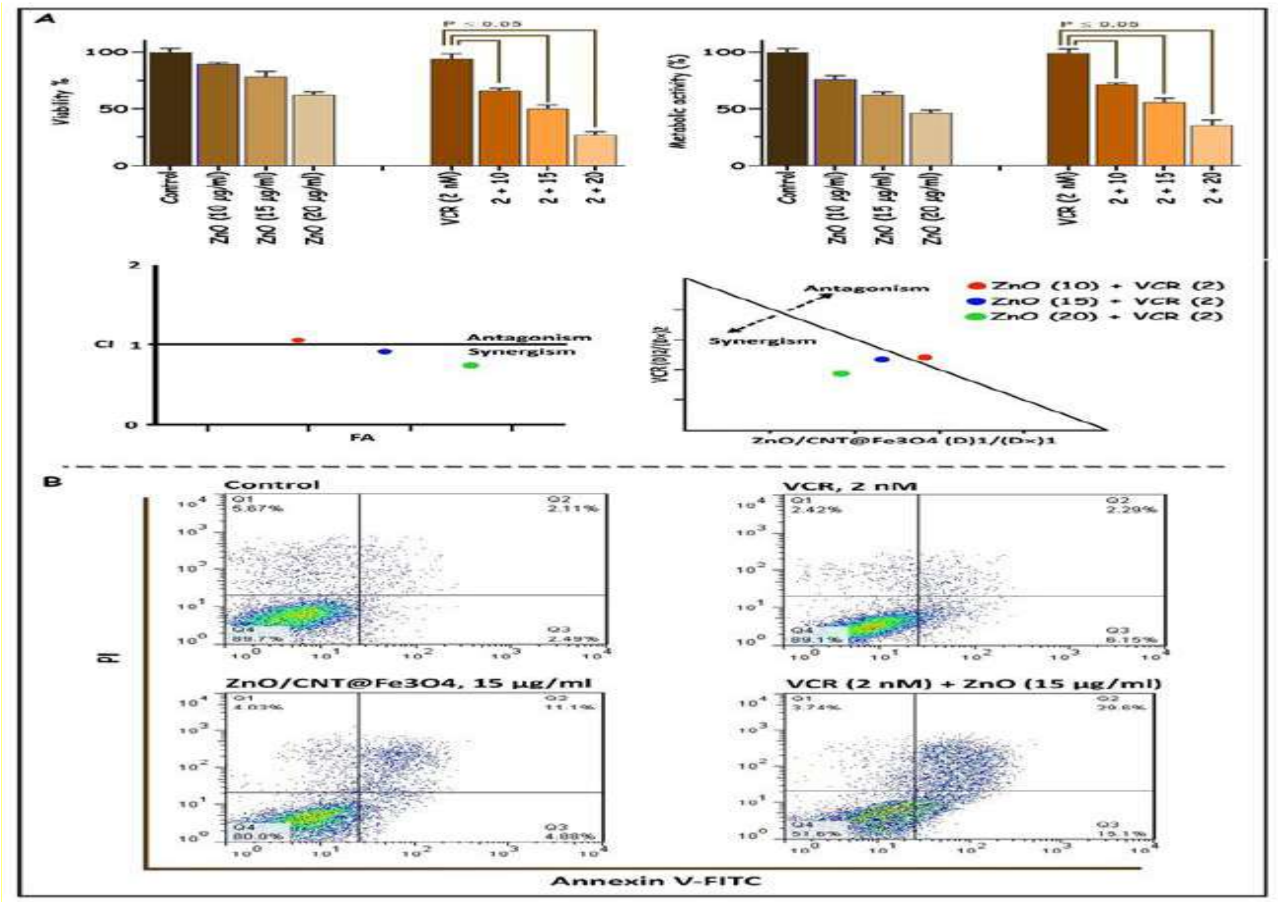

Figure 5. A) Combination of nanocomposite and VCR was more effective in reducing the survival of KG1 cells in comparison to each agent alone. The combination index (CI) was calculated according to the classic isobologram equation and the results revealed that ZnO/CNT@Fe_3O_4 at the concentrations of 15 µg/ml and 20 µg/ml and VCR reduced the viability of KG1 cells in a synergistic manner. Points above and below the iso-effect line reflect antagonism and synergism, respectively. **B)** The result of the Annexin-PI assay further confirmed the synergistic interaction between nanocomposite and VCR. Values are given as mean ± S.D. of three independent experiments. **P* ≤ 0.05 represented significant changes from the control.

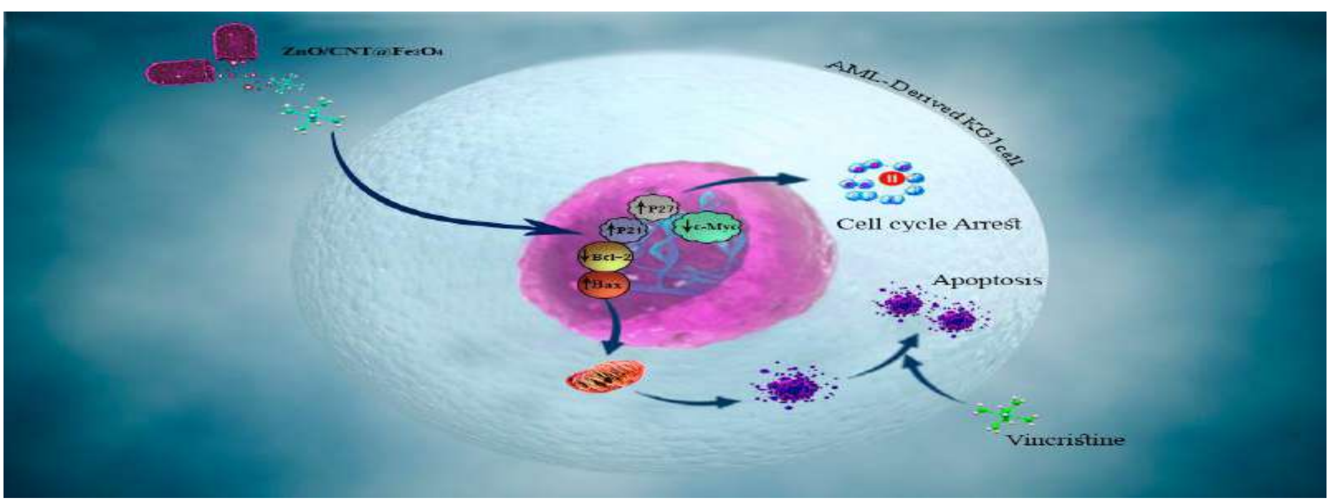

Figure 6. Schematic illustration proposed for the probable mechanism of action of ZnO/CNT@Fe_3O_4 in KG1 cells. Not only ZnO/CNT@Fe_3O_4 through alteration of the Bax-to-Bcl2 ratio induced apoptosis also exerted its antiproliferative effects on KG1 cells mainly through a p21- and p27-mediated G1 cell cycle arrest. Synergistic experiments also showed that the combination of ZnO/CNT@Fe_3O_4 and VCR could synergistically elevate apoptotic cell death in AML-derived KG1 cell line.

DISCUSSION

Therapeutic tactics for the treatment of AML remained unchanged for approximately 40 years and most of the patients will ultimately develop resistance and relapse[28,29]. Recently, novel nanoparticles (NPs) are rapidly progressing in both diagnostics and therapeutics fields by proposing merit solutions to many limitations of conventional drugs such as nonspecific targeting and low efficacy[30]. Among all the efficient NPs, most attentions have been focused on metal particles with an extensive focus on ZnO QDs and Fe_3O_4 NPs [31]. The favorable toxicity of zinc NPs for cancer cells and the potent ability of iron NPs to discriminate cancer cells from their normal counterparts encouraged us to bind these NPS to create a multifunctional nanocomposite system capable of killing cancerous cells while inducing minimal cytotoxicity against normal cells [32,33]. Moreover, because of low solubility of this NP in aqueous media, we used hydroxylated carbon nanotubes (CNT) in the combination of ZnO QD and Fe_3O_4 in the form of ZnO/CNT@Fe_3O_4 nanocomposites to increase their water-solubility. The chemical analysis, structure, morphology and size of ZnO/CNT@Fe_3O_4 nanocomposite were explored by FTIR, XRD, SEM, and TEM, respectively.

The result of our study demonstrated a significant decrease in viability and metabolic activity of AML derived-KG1 cells after treatment with ZnO/CNT@Fe_3O_4. Many studies revealed that the cytotoxicity of these nanomaterials, at least partially, may be induced as a result of reactive oxygen species (ROS) generation, which in turn can promote apoptosis signaling by inducing the expression of multiple pro-apoptotic members of the Bcl2-family of mitochondria-targeting proteins[14,34]. Interestingly, we found that ZnO/CNT@Fe_3O_4 induces apoptosis in KG1 cells through elevation of the ratio of the pro- to anti-apoptotic genes via alteration of Bax and Bcl-2 expression. Sirelkhatim et al. reported that ZnO NPs could provide signaling that in turn regulates cell cycle progression through induction of G1 or G2/M cell cycle arrest[35]. In agreement, we found that not only ZnO/CNT@Fe_3O_4 decreased the percentage of cells in the S phase but also blocked the transition of the cells from the G1 phase through increasing the expression levels of p21 and p27 cyclin-dependent kinase inhibitors. The resulting data also declared that treatment of KG1 cells with ZnO/CNT@Fe_3O_4 hindered the mRNA expression of c-Myc proto-oncogene, as the critical regulators of the cell cycle transition from G1 to S phase. In harmony, our recent study on chronic myeloid leukemia K562 cells indicates that the level of c-Myc expression was markedly decreased after treatment with ZnO/CNT@Fe3O4[14]. Moreover, intrigued by the results of a recent study which established the synergistic correlation between ZnO/CNT@Fe3O4 and imatinib on K562 cells[14] (Figure 6). We conducted synergistic experiment to determine the effects of this nanocomposite on the cytotoxic effects of VCR. Based on the results of combinatorial experiments, ZnO/CNT@Fe_3O_4 potentiated the cytotoxic effect of VCR against KG1 cells, as the viability and metabolic activity of the cells were lower in combinatorial series than either agent alone. Taken together, the results of the current study shed new light on the promising effects of metal nanoparticles in acute leukemia treatment and re-emphasized the fact that these molecules may have large impacts on cancer treatment strategies, either as a single agent or in combination with chemotherapeutic agents. However, further studies need to be carried out to confirm the safety and the usefulness of ZnO/CNT@Fe_3O_4 in cancer therapeutics.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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