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Cytotoxicity on V79 and HL60 Cell Lines by Thiolated-β-Cyclodextrin-Au/Violacein Nanoparticles

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In vitro cytotoxicity of a supramolecular system composed by violacein complexed by β-cyclodextrin-thiol-protected gold nanoparticles (violacein@β-CD–S$(CH_2)_6$–S–Au) on V79 and HL60 cell lines is described. The gold nanoparticles were prepared and modified in a single step involving reduction of tetrachloroaurate ions with sodium borohydride in the presence of thiol derivatized β-cyclodextrin. Inclusion complexation of violacein into cyclodextrin cavities occurred by mixing an aqueous solution of the gold nanoparticles with an acetone solution of violacein, being evidenced by UV/visible absorption spectroscopy. According to cell viability measurements based on the MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide) the supramolecular system was found to maintain the cytotoxic effects compared to free violacein on HL60 cells and in addition is less cytotoxic to normal (V79) cells.

Keywords: *In Vitro* Cytotoxicity, Gold Nanoparticles, Violacein, Antitumor, Drug Delivery, HL60.

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1. INTRODUCTION

Violacein (Fig. 1a), a purple-colored pigment produced by one of the strains of *Chromobacterium violaceum* found in the Amazon river, Brazil, is an indole derivative characterized as 3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one (Fig. 1).[1] The biosynthesis and biological properties of this pigment have been extensively studied; in particular, its antitumoral, antibacterial, antiulcerogenic, antileishmanial, and antiviral activities are of interest.[2–4] Its activity on myeloid leukemia cells (HL60) is of special interest, as the compound effectively induces cellular cytotoxicity in these cells. HL60 cells are generally accepted as a valid model for studying myeloid leukemia biology and Melo et al.[4] have earlier shown that this cell type reacts to violacein with both increased apoptosis and diminished cellular proliferation. Other results show that violacein mediates its cytotoxic effects in HL60 cells via an activation of TNF receptor signalling and that this effect is specific to myeloid leukemia cells as it is not observed in untransformed cells.[5] Recently, it was observed that violacein is at least three orders of magnitude more potent in human myeloid leukemia cells as compared to primary isolated human mononuclear cells obtained from healthy volunteers. Thus violacein effects resemble TNFα signal transduction in these cells. Hence, violacein mediates its cytotoxic effects in human myeloid leukemia cells via an activation of TNF receptor signalling specific to these cells, and an enhancement of this cytotoxic effect is of great importance in term of applying it in specific cancer cases.

Poor solubility of violacein in water, however, may cause low biological activity both *in vitro* and *in vivo*.[3] Previously, it was shown that modification in the physicochemical properties of violacein is achieved by the preparation of inclusion complexes with β-cyclodextrin (β-CD), Figure 1b, slightly increasing its cytotoxic effect on V79 cell line and significantly increasing the effect on HL60 cell line.[4] Natural cyclodextrins (CDs) constitute a family of cyclic oligosaccharides comprising repetitive 6, 7, or 8 glucopyranose units (α-, β-, and β-CD, respectively).[6] It is well known that complexation in β-CD can increase the

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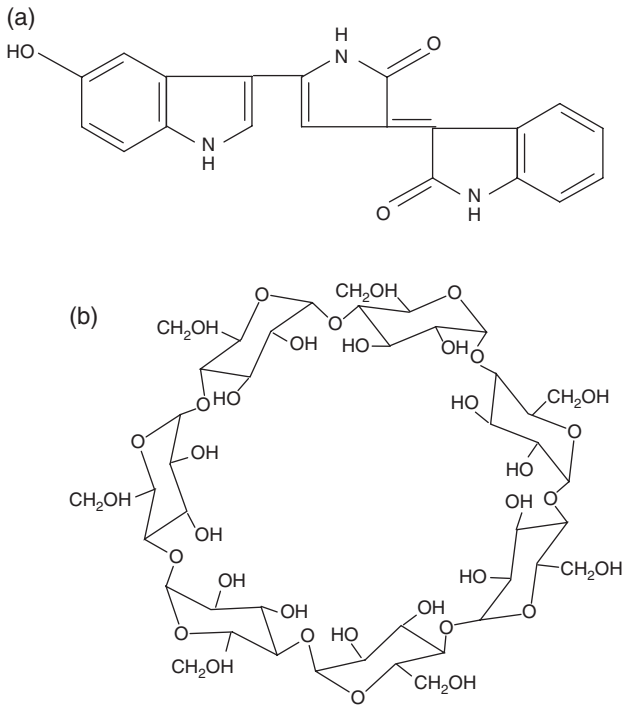

Fig. 1. Representations of (a) Violacein structure and (b) β-cyclodextrin structure.

solubility, stability, bioavailability, and the cell absorption of the guest molecule.[6]

Practical use of natural cyclodextrins, particularly that of β-cyclodextrin, as drug carriers is however restricted by its relatively low aqueous solubility, which can be overcome by structural modification of the parent cyclodextrin as well as the design of new formulations.[6] The recent burst of research involving gold nanoparticles as transfection vectors,[7] DNA-binding agents,[8] protein inhibitors,[9] and spectroscopic markers[10] demonstrates the versatility of these systems in biological applications. Gold particles display several features that make them well suited for biomedical applications, including straightforward synthesis, stability, and the facile ability to incorporate secondary tags such as peptides targeted to specific cell types to afford selectivity.[11]

Monolayers of mono-, multi-, and perthiolated cyclodextrin derivatives with different spacer length between the cavity and the thiol termination adsorbed on gold films have been prepared by a number of research groups.[12–19] Different thicknesses and packing densities of the molecular monolayer can be obtained by changing the number of thiol groups and the alkyl chain. The cyclodextrin cavities form the outermost layer of these systems and it is possible to control the orientation of the cyclodextrin cavity by varying the number of thiols anchors and the length of the alkyl chain. Concerning to cyclodextrin-modified nanoparticles, few reports by Kaifer and co-workers describe gold and palladium nanoparticles modified with per-6-thio-β-cyclodextrin,[15,16] a cyclodextrin derivative in which all secondary hydroxyl groups in the ring have been substituted by S—H groups. This method afforded the synthesis of highly water-soluble gold nanoparticles with the outermost layer formed by β-CD cavities. The use of a spacer group between the S—H terminations in the preparation of nanoparticles is novel and could increase the number of adsorbed β-CD molecules, improving particle size control as well.

In this work we report the preparation of nanoparticles modified by mono[6-deoxy-6-[(mercaptohexamethylene)thiol]]-β-cyclodextrin, a derivative with a medium spacer length in which only one secondary hydroxyl group has been substituted by 1,6-hexanedithiol. This research is also involved with the use of molecular recognition properties from the novel cyclodextrin-alkylthiol derivatives capping gold nanoparticles to complex the antitumor violacein and its cytotoxic properties on human leukemia cells (HL60) and lung fibroblast cells (V79) compared with violacein free form.

2. MATERIALS AND METHODS

The preparation of the supramolecular carrier can be summarized by the following steps:

β-cyclodextrin
→ monotosylated β-cyclodextrin (compound **1**)

monotosylated β-cyclodextrin (**1**)
→ thiolated β-cyclodextrin (compound **2**)

$HAuCl_4 + NaBH_4$ + thiolated β-cyclodextrin (**2**)
→ Au–S$(CH_2)_6$–S–β-CD (compound **3**)

Au–S$(CH_2)_6$–S–β-CD (**3**) + violacein
→ violacein@β-CD–S$(CH_2)_6$–S–Au (compound **4**)

Solvents and reagent grade β-cyclodextrin, p-toluenesulfonyl chloride, 1,6-hexanedithiol were purchased from Aldrich and used without further purification.

2.1. Thiolated β-Cyclodextrin

The compound (**1**) was prepared by mixing a solution of p-toluenesulfonyl chloride in dry pyridine with a solution of β-cyclodextrin in dry pyridine, cooled overnight below 5 °C. Then solvent was evaporated and the product was redissolved in diethyl ether.[20] To prepare compound (**2**) mono[6-deoxy-6-[(mercaptohexamethylene)thiol]]-β-cyclodextrin, compound (**1**) was dissolved in aqueous Na_2CO_3 (pH 12) containing 20% ethanol.[18] After complete dissolution, 1,6-hexanedithiol was added and the reaction mixture was heated under nitrogen at 50 °C for 5 h. The solution was acidified with HCl, washed with ether, neutralized with NaOH, acidified and extracted with trichloroethylene. The precipitate was filtered and dissolved in water. Concentration in vaccum followed by addition of water was repeated at least four times. The resulting aqueous solution was vaccum dried to give a white solid.

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2.2. Gold Nanoparticles

In a typical procedure for gold nanoparticles capped with the compound (**2**), a DMSO solution containing $NaBH_4$ was quickly mixed to another DMSO solution containing $HAuCl_4$ and compound (**2**) at room temperature.[15] The mixture was allowed to stir for 24 h and then the nanoparticle aggregates were precipitated by the addition of CH_3CN. The solid was isolated by centrifugation and washed three times with mixtures of CH_3CN:DMSO (1:1 v/v), then washed with ethanol and dried under vaccum. The nanoparticles were isolated as a deep brown solid which was found to be very soluble in water. The nanoparticles were characterized by TEM (Zeiss CEM-902), UV-Vis spectroscopy (HP 8452A diode array spectrophotometer in the range 190 to 820 nm with spectral resolution of 2 nm) and infrared spectroscopy of KBr discs (Bomen FTIR in the 4000–400 cm^{-1} range, with resolution of 4 cm^{-1}).

2.3. Violacein@β-CD–S$(CH_2)_6$–S–Au

Violacein (3-(1,2-dihydro-5-(5-hydroxy-1-H-indol-3-yl)-2-oxo- 3H- pyrrol- 3- ilydene)-1, 3- dihydro- 2H- indol- 2-one) was isolated from *C. Violaceum* and purified as previously described.[21] In the violacein@β-CD–S$(CH_2)_6$–S–Au (**4**) preparation an aqueous solution of Au–S$(CH_2)_6$–S–β-CD nanoparticles (**3**) was mixed with an acetone solution of violacein.[22, 23] The mixture was stirred and heated at 60 °C for 2 h and then the acetone was evaporated. A residue of violacein was precipitated and separated from the aqueous phase by centrifugation. The aqueous solution was lyophilized and the amount of violacein incorporated in the solid was determined by elemental analysis.

2.4. Cell Culture

Human leukemia cells (HL60) were cultured[24] in suspension in RPMI 1640 medium (Sigma Chemical Co., MO) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin in a humidified atmosphere at 37 °C in 5% CO_2. Cells were seeded (3.10^5 cells/ml) in 96-wells and 35-mm plates, and incubated with different concentrations of violacein or violacein@β-CD–S$(CH_2)_6$–S–Au for 72 h. Cell viability was determined by the MTT reduction test.

V79 fibroblasts cells derived from Chinese hamsters were grown as mono layers in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU:ml penicillin and 100 mg:ml streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cells were plated at density of 3.10^4 per ml in 96-well plates. The medium was removed 48 h after cell seeding and replaced with medium containing the compounds in study. Cells were exposed for 24 h to test medium with or without violacein derivatives (control). Each drug concentration was tested in four replicates and repeated three times in separate experiments. At the end of incubation, endpoints for cytotoxicity were evaluated from MTT.

2.5. MTT Assay

The cytotoxic effects of the compounds (violacein, Au–S$(CH_2)_6$–S–β-CD and violacein@β-CD–S$(CH_2)_6$–S–Au) were measured by MTT reduction assay on V79 and HL60 cells. The tetrazolium reduction assay was performed according to the method of Denizot and Lang.[25] Briefly, cells were washed once with PBS before adding 0.1-ml serum-free medium containing MTT (1mg:ml) to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1 ml ethanol with stirring for 15 min on a microtitre plate shaker and the absorbance was read at 570 nm.

3. RESULTS

Nanoparticle samples are spherical shaped as can be observed in the TEM images (Fig. 2a). Figure 2b presents the hystogram plot for particle diameter distribution, determined by measuring the size of about 500 particles, which shows an approximate unimodal behavior. The average particle diameter determined from the size analysis was 3.8 ± 1 nm.

The presence of the cyclodextrin derivatives was indicated by the FTIR spectra (KBr discs) of the nanoparticles isolated as solids, which show bands characteristic of the cyclodextrin rings (Fig. 3). In comparison with the spectrum of the free compound (**2**), the intensities of bands at 2923 and 2852 cm^{-1}, assigned respectively to the asymmetric and symmetric CH_2 stretchings from the spacer chain, are strongly reduced. The disappearance of a band at 2921 cm^{-1} assigned to asymmetric CH_2 stretching has also been observed upon binding of thiolated cyclodextrin derivatives onto the surface of gold films. In a previous study[18] the observed peak shifts and intensity changes have been interpreted on the basis of the fact that IR spectra of immobilized thiols contain only those vibrations with a dipole moment component perpendicular to the gold surface.[17, 18]

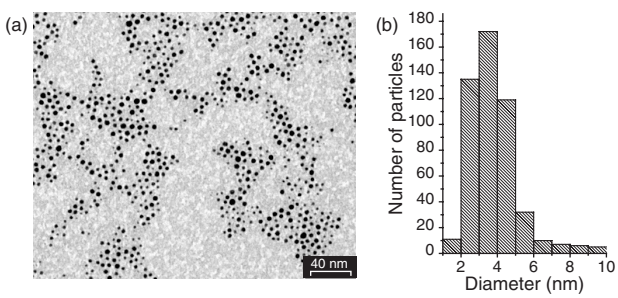

Fig. 2. (a) TEM image of the gold nanoparticles; (b) hystogram plot for the distribution of diameters determined by measuring the size of about 500 particles.

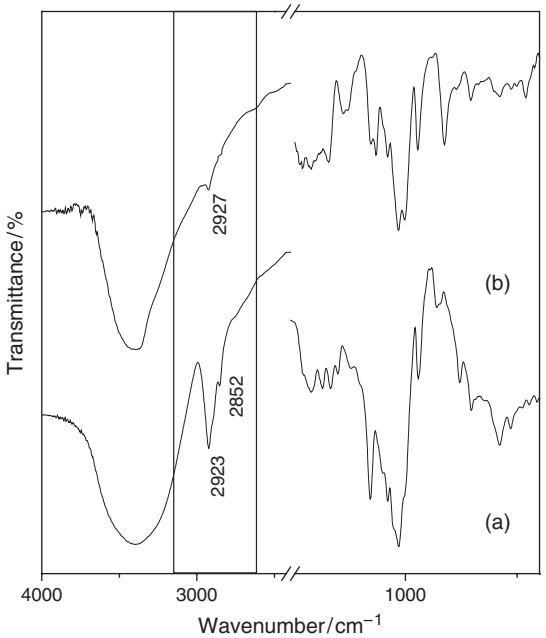

Fig. 3. FTIR spectrum of KBr discs of (a) compound (**2**); (b) Au–$S(CH_2)_6$–S–β-CD.

The UV/visible absorption spectrum of the resulting material in water (Fig. 4a) presents a characteristic violacein shoulder at 224 nm and a band centered at 530 nm. This is in contrast to the reported spectrum of violacein ethanolic solution,[21] which presents a band centered at 580 nm and the spectrum of the Au–$S(CH_2)_6$–S–β-CD nanoparticles, which presented a surface plasmon resonance absorption at 516 nm (Fig. 4b).

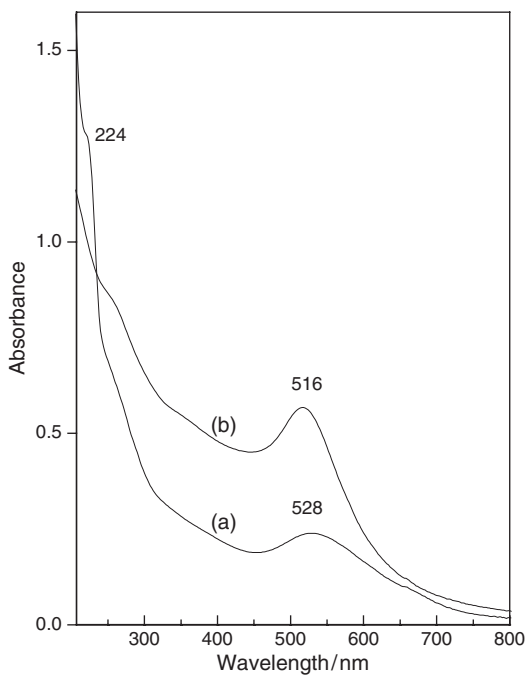

Fig. 4. UV/visible absorption spectrum of diluted aqueous solutions of (a) Au–$S(CH_2)_6$–S–β-CD (**2**); (b) violacein@β-CD–$S(CH_2)_6$–S–Au.

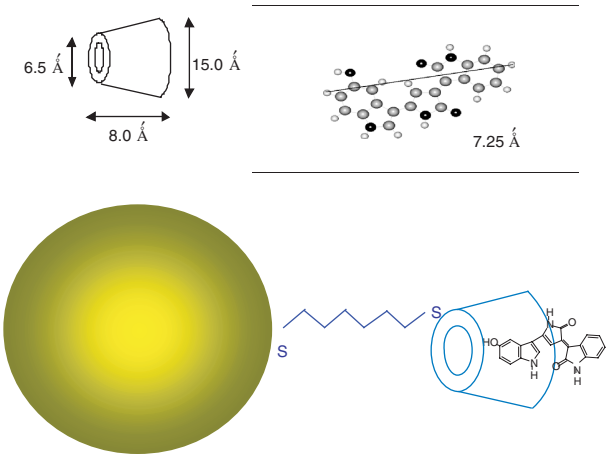

Fig. 5. Comparative sizes of violacein and cyclodextrin cavities and schematic structure of the whole inclusion complex violacein@β-CD–$S(CH_2)_6$–S–Au.

The violacein content was also measured by elemental analysis: 1.017 mg of violacein@β-CD–$S(CH_2)_6$–S–Au contain 16.80% in C, 3.33% in H, and 1.30% in N. Therefore, there is 10.63% of violacein in the violacein@β-CD–$S(CH_2)_6$–S–Au complex. A schematic assemblage of the resulting material is proposed in Figure 5, where a comparison of cyclodextrin/violacein relative sizes can also be found.

3.1. Effect on Cell Viability

Viable cells have the ability to reduce MTT from a yellow water-soluble dye to a dark blue insoluble formazan product. Formazan crystals were dissolved in ethanol and quantified by measuring the absorbance of the solution at 570 nm, and the resultant value is related to the number of

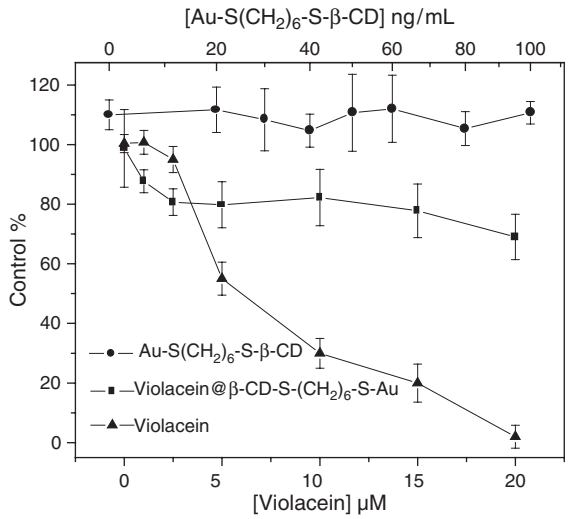

Fig. 6. Viability of V79 cells after a 24 h treatment with Au–$S(CH_2)_6$–S–β-CD, violacein or violacein@β-CD–$S(CH_2)_6$–S–Au, evaluated by MTT reduction assay. Each point represents the mean ± SD of two experiments with four replicates.

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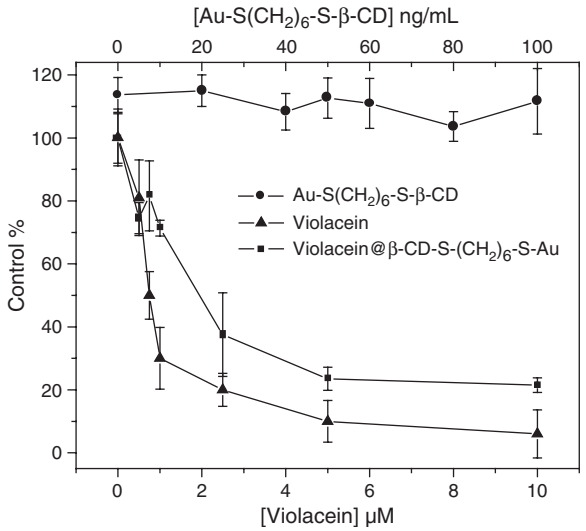

Fig. 7. Viability of cultured HL60 cells after treatment with Au–$S(CH_2)_6$–S–β-CD, violacein or violacein@β-CD–$S(CH_2)_6$–S–Au for 72 h, evaluated by MTT reduction assay. Each point represents the mean ± SD of two experiments in four replicates.

living cells.[25,26] The cytotoxic effect of the compounds, expressed as cell viability, was assessed on a permanent lung fibroblast cell line derived from Chinese hamsters (V79), which is commonly used for cytotoxicity studies since it is well characterized and human leukemia cells (HL60).[27] In both cell cultures the Au–$S(CH_2)_6$–S–β-CD was not cytotoxic at the maximum concentration evaluated (100% of cell viability at 100 ng/ml) demonstrating the absence of cytotoxic effects of the pure complex (Figs. 6 and 7). However in V79 cells (Fig. 6) the violacein@β-CD–$S(CH_2)_6$–S–Au showed less toxicity in the MTT assay than free violacein, which reached a 50% inhibitory effect on the cell viability (IC_{50}) at the concentration of 5 μM. These results are in agreement with data of Melo and collaborators[28] showing the cytotoxic effects of violacein and the induction of apoptosis on V79 cells and indicate that the complexation of violacein reduces its cytotoxicity to normal cells.

In contrast, the free violacein in HL60 cells exhibited an IC_{50} of 0.8 μM and the violacein@β-CD–$S(CH_2)_6$–S–Au showed an IC_{50} of 1.8 μM (Fig. 7). These results indicate that the complexation of violacein with Au–$S(CH_2)_6$–S–β-CD maintained the cytotoxicity. It is important to emphasize that the treatment of V79 and HL60 cells with the Au–$S(CH_2)_6$–S–β-CD (in the absence of violacein) did not induce any cytotoxic effects on these cells at the concentrations used.

4. DISCUSSION

Conventional anticancer therapies face problems associated with distribution, biotransformation and clearance of anticancer drugs in the body.[29] First, non-cellular drug resistance mechanisms could be due to poorly vascularized tumor regions which can effectively reduce drug access to the tumor, as well as the pH conditions and interstitial pressure of the neoplastic tissue.[30] On the other hand, the resistance of tumors to therapeutic intervention may also be due to cellular mechanisms, which are categorized in term of alterations in the biochemistry of malignant cells.[31] They comprise altered activity of specific enzyme systems, altered apoptosis regulation, or transport based mechanisms. The use of nanoparticles in anticancer therapy is based mainly on the association of the conventional drugs with nanoparticle carriers, in order to protect the molecules from premature inactivation during transport, to increase drug concentration in cancer tissues as well as to reduce their toxicity towards normal tissues.[29] In an alternative approach for cancer treatment, it was recently reported that gold nanoparticles bind and inhibit the activity of vascular endothelial growth factor 165 (VPF/VEGF-165), a prime mediator of angiogenesis that plays a role in pathological neovascularization including neoplastic disorders.[32] The use of gold nanoparticles was also reported as an indicator for immunodiagnostics,[33] conjugation with DNA,[34] proteins,[35] to list a few subjects.

In this work, violacein, β-cyclodextrin, 1,6-hexanedithiol and gold nanoparticles were properly assembled to obtain a supramolecular system for *in vitro* induction of apoptois in myeloid leukemia cells. The supramolecular system was designed in order to exploit the physicochemical and biological properties of the various components: the β-cyclodextrin can host drugs of low solubility and stability, 1,6-hexadithiol was used as a spacer between β-cyclodextrin and the gold surface in order to confer to the target moiety a certain flexibility that overcome problems related to steric hindrance of cyclodextrin. The monolayer protected cluster Au–$S(CH_2)_6$–S–β-CD was found to maintain high affinity for violacein.

First it was of fundamental importance to be sure that the monolayer protected cluster Au–$S(CH_2)_6$–S–β-CD induced no cytotoxic effects on the cells. Cytotoxicity of mixed monolayer protected gold clusters has been described at high concentrations using mixed cationically functionalized systems and was associated with the nature of the molecular monolayer.[36] Here, cell culture studies demonstrated that monolayer protected cluster Au–$S(CH_2)_6$–S–β-CD (in the absence of violacein) was devoid of toxicity to both the cell lines at the concentrations studied. High cell viability was, in fact, observed by incubating both V79 and HL60 with high concentrations of Au–$S(CH_2)_6$–S–β-CD, ensuring that any cytotoxic effect of the violacein loaded system can be assigned to the presence of violacein.

In the present study, it was found that the cytotoxicity of the violacein loaded system violacein@β-CD–$S(CH_2)_6$–S–Au on HL60 cells is similar to that of free violacein. On the other hand, the system violacein@β-CD–$S(CH_2)_6$–S–Au presented a significative decrease in the cytotoxicity on

V79 cells, indicating a degree of specificity to the malignant cells studied. Results previously described for 1:1 and 1:2 violacein/β-CD complexes not bound to gold nanoparticles demonstrated that inclusion complexation in the β-CD cavities did not reduced the cytotoxicity of violacein on V79 cells.[37, 38] Since free β-CD showed no activity over the concentration range used, the cytotoxicity on V79 cells can be assigned to the uptake of violacein by the cells, whether free or complexed still remains unknown. In the present case the enhanced specificity seems to be due to the fact that cellular uptake of the system violacein@β-CD–S$(CH_2)_6$–S–Au can only take place for the HL60 cells but further evidences are necessary in order to be conclusive about this point.

The mechanism of action of the complex violacein@β-CD–S$(CH_2)_6$–S–Au is probably very similar as violacein-@β-CD. Recently, Haun and co-workers reported that cell death was caused by violacein and its complexes in HL60 due to apoptosis.[5] Violacein cytotoxicity in HL60 cells was preceded by activation of caspase 8, transcription of nuclear factor kappaB (NF-kappaB) target genes, and p38 mitogen-activated protein (MAP) kinase activation. Thus, violacein effects resemble tumor necrosis factor alpha (TNF-alpha) signal transduction in these cells. Hence, violacein is a member of a class of cytotoxic drugs mediating apoptosis of HL60 cells by way of the specific activation of TNF receptor. Recently Paciotti and co-workers described a similar drug carrier based on Au nanoparticles suitable for *in vivo* use that targets the delivery of tumor necrosis factor (TNF) to a solid tumor growing in mice.[39] The carrier, designated PT-cAu-TNF, consisted of molecules of thiol-derivatized PEG (PT) and recombinant human TNF that are directly bound onto the surface of the gold nanoparticles.

Concerning to *in vivo* application of the system violacein@β-CD–S$(CH_2)_6$–S–Au, it is premature to suggest the effects and the stability of the complex as well. This is a preliminary study and long termed effects on specific cellular function in order to state the safety of those compounds cannot be concluded from this single assay.

5. CONCLUSIONS

In conclusion, it was demonstrated that inclusion complexation of violacein in β-CD–S$(CH_2)_6$–S–Au gold nanoparticles (3.8 nm diameter) afforded violacein transfer to aqueous medium. Cell viability measurements indicated that the system violacein@β-CD–S$(CH_2)_6$–S–Au maintained the cytotoxicity of violacein on myeloid leukemia HL60 cells, reducing the activity on normal V79 cells, in contrast to other violacein/β-CD complexes previously described.

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