Hairpin Oligonucleotide Can Functionalize Gold Nanorods for in Vivo Application Delivering Cytotoxic Nucleotides and Curcumin: A Comprehensive Study in Combination with Near-Infrared Laser

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ABSTRACT: We prepared a multimodality nanocomplex by functionalizing gold nanorods (GNRs) with a cytotoxic nucleoside, 5-fluoro-2′-deoxyuridine (FdU) containing a DNA hairpin, along with complexation of pleiotropic molecule curcumin. Conjugates were investigated for anti-tumor activity using an Ehrlich carcinoma model in combination with 808 nm laser irradiation. We demonstrated that hairpin-functionalized GNRs are suitable for intravenous administration, including delivery of cytotoxic nucleotides and curcumin. Curcumin binding with FdU-hairpin-functionalized GNRs displayed improved anti-tumor activity in part by inducing a lymphocyte-mediated immune response. The complex showed notable photothermal activity in vitro; however, 808 nm laser irradiation of the tumor following treatment with the complex did not increase the anti-tumor effect significantly. Biodistribution studies depicted that the nanoconjugates localized primarily in the sinusoidal structures of the liver and spleen with minimal tumor accumulation. Curcumin complexation alleviated the reduction in the RBC count that was observed for the conjugate without curcumin, especially in combination with laser irradiation. Localization of FdU-hairpin-GNR conjugates in the liver and spleen evoked an inflammatory response, which was mitigated by curcumin complexation. However, no functional abnormality was found in the liver in any case. Curcumin binding also notably decreased nanoconjugate accumulation in lungs and significantly reduced inflammation. Biodistribution studies were consistent with previous reports, suggesting that optimization of the GNR size and surface coating is required for more efficient tumor localization via the enhanced permeability and retention (EPR) effect. Our studies demonstrate that DNA/RNA hairpins are suitable for GNR surface functionalization and enable delivery of cytotoxic nucleotides as well as curcumin in vivo with potential for synergistic anti-cancer therapy.

INTRODUCTION

Selective multimodality treatment is an emerging technique for treating patients with advanced malignancies using a less invasive approach.1−4 In advanced stages of cancer, significant numbers of malignant cells survive therapeutic measures due to drug resistance, contributing to metastasis.5,6 Therefore, there is an urgent need for progressive strategies to treat advanced cancer by overcoming chemoresistance and simultaneously reducing systemic toxicities.7 Combined application of multiple modalities could enhance killing of chemoresistant cells, potentially without increasing systemic toxicities.2,6,8 Gold nanorods (GNRs) are well known for their photothermal activity and inherent tumor targeting property via the enhanced permeability and retention (EPR) effect.9,10 GNRs are now being developed as a suitable tool for constructing multimodality therapeutics.11,12

Translational applications of GNRs remain limited due to challenges with its surface functionalization.13,14 Extensive studies have investigated the exchange of the GNR surface coating with biocompatible molecules including polyethylene glycol (PEG), peptides, and oligonucleotides.1,1,15,16 The PEG coating results in an incomplete ligand exchange, while peptide conjugates display limited stability and dispersity in biologically relevant conditions.17,18 Functionalization of GNRs with DNA has been studied by several research groups,13,19,20 and in contrast with other biocompatible molecules, oligonucleotides could efficiently exchange with the overall surface coating. Further, DNA bases may be substituted with cytotoxic nucleotides to enhance the anti-tumor activity and DNA can form various secondary structures that enable intercalation of anti-neoplastic agents.21,22 In our previous studies, we
demonstrated that DNA hairpins could optimally functionalize the GNR surface without reducing its near-infrared (NIR) absorbance, while single- as well as double-stranded DNAs caused severe aggregation, including a major decrease in NIR absorbance. The resulting nanconjunctures are suitable for intracellular uptake and remain monodispersed in the cellular environment. In a separate study, we also reported that the DNA hairpin could form stable, non-covalent complexes with curcumin that displayed notable biological activity. 

While earlier studies reported several applications for DNA-conjugated GNRs in vitro, the in vivo efficacy of these complexes has never been studied for further translational evaluation. In the present study, we developed a multimodality nano-conjugate via functionalizing the GNR surface with a cytotoxic DNA hairpin followed by hydrophobic complexation of curcumin (Figure S1, Supporting Information). We used a cytotoxic nucleoside, 5-fluoro-2′-deoxyuridine (FdU), containing a hairpin for the GNR surface coating via Au–S bonding, while curcumin was bound in the hydrophobic environment of the GNR surface as well as in the hairpin minor groove (Figure S2, Supporting Information). FdU-containing oligonucleotides are well-known cytotoxic modalities having metabolic advantages over their clinically used counterpart, 5-fluorouracil. Moreover, FdU hairpins should play multiple roles in our strategy, including GNR surface functionalization, exerting cytotoxic activity and providing a suitable environment for curcumin binding. The pleiotropic molecule curcumin could reduce the requirement of cytotoxic agents as well as the magnitude of hyperthermia necessary to achieve a beneficial anti-tumor effect, enhancing the multimodality approach.

We further investigated the biodistribution, toxicity, and anti-cancer activity of the nanoconjugates in combination with NIR laser in an Ehrlich carcinoma model. The conjugate was well tolerated in vivo and displayed moderate anti-tumor activity; however, 808 nm laser irradiation showed no synergistic photothermal effect. Atomic absorption spectroscopy demonstrated maximum gold accumulation in the liver followed by the spleen and lungs with limited accumulation in the tumor, consistent with GNR-size and coating optimization being required for more efficient tumor localization. Curcumin complexation countered the decrease in hemoglobin levels and reduced the pulmonary inflammation that was induced by FdU-hairpin-GNR treatment. Our findings demonstrate that the hairpin oligonucleotide could functionalize GNRs suitably for in vivo applications, delivering cytotoxic nucleotides and chemosensitizing agents for synergistic cancer therapy; however, the size distribution and coating need optimization to achieve an EPR effect.

 RESULTS AND DISCUSSION

Biophysical Characterization of the Conjugates.

Analysis of UV–visible spectra (Figure 1a) showed that the NIR band of the cetyltrimethylammonium bromide (CTAB)-coated GNR is red-shifted from 791 to 810 nm upon DNA conjugation. A characteristic DNA band was observed in all FdU-hairpin-functionalized GNR samples. Curcumin complex formation changed the color of the suspension from reddish pink to yellowish brown (Figure S2a, Supporting Information) and significantly reduced the NIR band intensity by ∼18%. Curcumin complexes displayed a characteristic peak near 425 nm, indicating successful complexation. A red shift in the NIR band could have resulted from lower interferences of FdU-hairpin bases with the GNR surface, enhancing its surface plasmon resonance. Curcumin is complexed with FdU-hairpin-GNRs via a noncovalent interaction, where it has the opportunity to interact with the hairpin minor groove as well as the hydrophobic GNR surface. The close interaction between curcumin and the GNR surface leads to damping of plasmon
resonance, resulting in NIR peak broadening and reduced band intensity. Fluorescence spectroscopy showed no band in the region of 450–650 nm in the FdU-hairpin-GNR sample, while curcumin binding resulted in a small peak at ~490 nm. The characteristic fluorescence peak of curcumin at 535 nm is not observed due to the quenching effect of the GNR, which absorbs at 500–600 nm because of its transverse plasmon resonance (Figure 1b). Dynamic light scattering (DLS) spectroscopy showed that the majority of the FdU-hairpin functionalized GNRs have hydrodynamic diameters in the range of 37.84–78.82 nm, while curcumin complexation shifted the hydrodynamic diameter to some extent toward the higher range, 50.75–91.28 nm (Figure 1c). Figure S3a, Supporting Information, also depicts a similar pattern of size distribution based on the scattering intensity. The presence of a single distribution in both cases with majority of the particles suggests no significant agglomeration.27,28 The magnitude of the shift in the hydrodynamic diameter distribution is not enough to indicate aggregation; rather, it could be due to a size increase because of curcumin intercalation.29–31 The zeta potential changed from +39.3 to −28.5 mV for the FdU-hairpin conjugation (Figure S3b, Supporting Information), suggesting a successful exchange of the GNR coating and removal of CTAB. In the case of curcumin complexation, the zeta potential was measured at −22.2 mV. A higher-magnitude zeta potential of the FdU-hairpin-GNRs suggested better stability in suspension, while curcumin binding reduced the repulsion between GNR conjugates to some extent. However, the resulting suspension should be sufficiently stable for further applications.16,32,33 Dispersion of the nanocojungates was further investigated using transmission electron microscopy (TEM). The micrographs depicted well-dispersed FdU-hairpin-GNR conjugates, while curcumin binding did not show significant changes in dispersion (Figure 1d,e).

Evaluation of Photothermal Effect in Vitro. The heating efficiency of nanoconjugates was evaluated to determine the role of FdU-hairpin functionalization and curcumin complexation on the photothermal effect of GNR. Aqueous suspensions of 150, 300, and 600 μg/mL of concentration were irradiated by an 808 nm continuous laser beam with power of 1.0, 1.5, and 2.0 W for 30, 60, and 120 s. Representative graphs displaying the concentration, time, and laser power-dependent heating are exhibited in Figure 2a–c, respectively. All comparisons had p values less than 0.05. The concentration dependence of the photothermal effect of GNR conjugates followed a nonlinear pattern (Figure 2a) when irradiated at 1.5 W for 60 s. Compared to the CTAB coating, the FdU-hairpin conjugation caused a minute increase while curcumin complexation resulted in an ~20% decrease at a 300 μg/mL concentration.32 FdU-hairpin-functionalized GNRs should achieve biologically relevant hyperthermia with application of optimum laser power and time of irradiation. For example, 300 μg of CTAB- and FdU-hairpin-coated GNRs can increase the temperature of 1 mL of water by ~8 °C when irradiated with a 1.5 W laser for 60 s, while curcumin-complexed GNR conjugates cause an increase of 6.2 °C with identical parameters. Previous studies reported that hyperthermia-induced malignant-cell death could be achieved above 42 °C.34,35 The efficiency of heat-induced cell death could be further enhanced with simultaneous application of suitable cytotoxic agents, such as FdU and curcumin.32 Figure 2b depicts the linear, time-dependent heating of GNR conjugates for a given concentration and laser power, which is informative to verify the attainment of therapeutically relevant hyperthermia.

For example, 90 s irradiation of 300 μg of CTAB- as well as FdU-hairpin-coated GNRs can increase the temperature of 1 mL of water by 11 °C with 1.5 W laser power, whereas curcumin-complexed FdU-hairpin-GNRs caused a 5 °C temperature increase with similar parameters. However, combining multiple cytotoxic agents may reduce the extent of heating required to induce cell death. The laser power-dependent heating shows an exponential pattern (Figure 2c). While 1.5 W laser irradiation for 60 s can increase the temperature of 1 mL of water by 7.5 and 9.0 °C for 300 μg of CTAB- and FdU-hairpin-coated GNRs, respectively, a 6.2 °C temperature increase was observed with curcumin complexation in similar conditions. Thus, FdU-hairpin-functionalized GNRs with and without curcumin binding should be able to achieve biologically effective photothermal activity, utilizing available parameters. Moreover,
Elevated hyperthermia could be obtained in the tumor microenvironment, compared to in vitro conditions.36,37

Evaluation of Anti-tumor Activity. Eradication of tumor tissue using a multimodality approach is an important objective in cancer research, especially in chemoresistant diseases. To investigate the potential synergistic anti-tumor activity of our GNR conjugates, we developed solid tumors in the neck region of Swiss albino mice (Figure S4a, Supporting Information). Initial tumor volumes were ~100 mm³. Animals were treated by tail-vein injection of either of the different nanoconjugates, such as FdU-hairpin-GNR (FG), FdU-hairpin-GNR:curcumin (FGC), or sterile water (NT).38 Each nanoconjugate-treated group was further divided into laser-treated and mock-treated groups to create six groups as mentioned in the Methods section. Laser treatment was done by continuously irradiating the tumor for 90 s with an 808 nm laser beam of 1.5 W/cm², as shown in the video clip of the associated content. The time-dependent tumor growth is shown in Figure 3a. Beginning around day 7, tumors treated with FGC and its laser combination (FGCL) displayed slower tumor growth, while no-treatment (NT) and laser-only (L) groups continued the rapid growth rate; the difference in growth rates was statistically significant (p < 0.05; Table S1, Supporting Information) until conclusion of the study. FG with and without laser irradiation also showed higher growth rates compared to FGC and FGCL, starting around day 14, and they remained statistically significant until the end of the study (p < 0.05). There was no statistically significant difference in tumor growth for FG-treated animals in the presence or absence of the laser with the no-treatment or laser-only group. The results demonstrate that curcumin-complexed FdU-hairpin-GNRs showed moderate anti-tumor activity, while a GNR-mediated photothermal effect was not observed with laser irradiation. Figure 3b depicts a graphical representation of the tumor weight after completion of the treatment and follow-up period. The results are consistent with the change in the tumor volume. Compared to the no-treatment group, only FGC and FGCL showed statistically significant reduction in tumor weight (Table S2, Supporting Information). FdU-hairpin-GNR treatment and its laser combination obtained no significant reduction in the tumor weight when compared to control groups. Laser irradiation showed no additional change in the tumor weight in any group. In Figure 3c, photographic images of representative tumors isolated from different experimental groups showed longer dimensions with the help of a caliper. Body weights of all animals were measured weekly, beginning from the day of tumor cell inoculation. Animals did not show any significant reduction in body weight due to nanoconjugate treatment (Figure S4b, Supporting Information) until completion of the study.

The cytotoxicity of FdU-hairpin-functionalized GNRs was also investigated in HeLa cells with and without curcumin complexation and in the presence or absence of 808 nm laser irradiation (Figure S5, Supporting Information). It is evident from the data that FdU-hairpin-coated GNRs demonstrated cytotoxicity in a dose-dependent manner at concentrations ranging from 93.75 to 3000 ng/mL. Curcumin complexation further enhanced the cytotoxic effect in all cases. However, 808 nm laser irradiation caused no additional cytotoxic activity.

Investigation of the Biodistribution and Blood Biochemistry. Figure 4a depicts the distribution of gold in the tumor tissues and vital organs, such as the liver, kidney, lung, heart, and spleen. The biodistribution was investigated by atomic absorption spectroscopy after 4 weeks of treatment and 2 weeks of a follow-up period. The liver and spleen showed maximum accumulation of gold in the range of 1.0–1.4 μg/g followed by the lungs and heart with 0.3–0.7 and 0.2–0.4 μg/g.
concentrations of gold in tumor tissue suggested that the fenestrated structure of capillaries.39 The endothelium of the kidney glomeruli acts as a barrier for the GNR.39 Very low concentrations of gold in tumor tissue suggested that the gold accumulation in the liver and spleen could be attributed to their fenestrated structure of capillaries.39 The endothelium of the kidney glomeruli is also known to be fenestrated. However, the increased neutrophil percent is consistent with an inflammatory response.45,46 The lymphocyte population followed the reverse pattern compared to neutrophils with FG lowering lymphocyte levels and the curcumin complex formation countering the reduction. Statistical analysis of changes in neutrophil and lymphocyte populations is depicted in Tables S10 and S11, Supporting Information. FACS analysis of blood samples, probing CD4 and CD8 markers, also suggested an increase in the T-lymphocyte population for curcumin-bound FdU-hairpin-GNR treatment and its laser combination. Further, flow cytometry data revealed elevated levels of NK cells with curcumin complexation (Figure S6, Supporting Information).

Histopathological Investigation. Histopathological investigation of tumors along with the vital organs from all experimental groups was done by analyzing hematoxylin and eosin (H&E) stained sections. Both control and treatment groups showed tumor tissue invasion and, for all groups, the tumors exhibited a high degree of cellular proliferation, as indicated by the mitotic index (data not shown). The renal histopathology results indicated that there were no significant changes between the groups (Figure S7, Supporting Information). Liver sections from all groups showed normal histological features, with no evidence of fibrosis or cirrhosis. The spleen sections showed no evidence of splenomegaly or infiltration with lymphocytes. The heart sections showed no evidence of myocardial fibrosis or hypertrophy. The lung sections showed no evidence of interstitial fibrosis or alveolar hyperplasia.

Figure 4. Biodistribution of gold and blood biochemistry. (a) Graphical representation depicting the biodistribution of Au using atomic absorption spectroscopy. Maximum accumulation was observed in the cases of the liver and spleen followed by that of the lungs, whereas low levels of gold were found in the tumor, kidney, and heart. FdU-hairpin-GNR-treated animals (FG and FGL) showed greater accumulation of gold in the lungs compared to groups with curcumin complexation (FGC and FGCL). Results are expressed as means ± SEM, n = 6. Statistical analysis was done using a one-way ANOVA test by Tukey’s multiple comparison method; p < 0.05 was considered statistically significant in all cases. ***, #, Δ, and ϕ are designated to FG vs FGC, FG vs FGCL, FGL vs FGC, and FGL vs FGCL, respectively. (b) Graphical representations of aspartate aminotransferase (AST) activity and alkaline phosphatase (ALP) activity in serum and serum creatinine and urea levels. Results showed no significant change in any case. Results are expressed as means ± SEM, n = 6. Statistical analysis is done using a one-way ANOVA test by Tukey’s multiple comparison method (p ≤ 0.05).
were further recon some extent with and without laser irradiation. The observations and FdU-hairpin-GNR:curcumin could induce apoptosis to arrows. Histological analysis indicates both FdU-hairpin-GNR also detected in these treatment groups, marked with yellow presence and absence of a laser. Patches of necrotic regions were circles, were detected in all nanoconjugate-treated groups in the intensely eosinophilic cytoplasm, as marked by black dashed circles, were detected in all nanoconjugate-treated groups in the presence and absence of a laser. Patches of necrotic regions were also detected in these treatment groups, marked with yellow arrows. Histological analysis indicates both FdU-hairpin-GNR and FdU-hairpin-GNR:curcumin could induce apoptosis to some extent with and without laser irradiation. The observations were further confirmed by immunohistochemical staining.

Figure 5. Histopathological investigation of H&E-stained liver tissue sections. The no-treatment group (NT) demonstrated normal hepatic parenchyma. The laser-only group (L) shows similar histology to NT. In the FdU-hairpin-GNR-treated group (FG), severe inflammation with lymphocytic infiltration (indicated with black arrows) and fibrosis was observed around the portal triad. A similar observation was found in the FdU-hairpin-GNR + laser group (FGL) without any sign of fibrosis. The curcumin-complexed groups with and without a laser (FGCL and FGC) showed lesser lymphocytic infiltration (shown with black arrows). Magnification: 200×, scale bar: 50 μm.

Figure 6. Histological investigation of tumor sections from different treatment groups. The NT group depicted massive infiltration of mononuclear cells, marked using black arrows, around a portal triad along with signs of fibrosis. These infiltrates majorly comprise focal aggregates of lymphocytes, plasma cells, and macrophages, accumulated due to inflammation resulting from nanoconjugate buildup.37 Meanwhile, fibrosis represents a reparative process to replace injured cells, reflecting moderate levels of damage in hepatic parenchyma.38 A closer view of the lymphocytic infiltration around the central vein is depicted in Figure S7 of the Supporting Information with 400× magnification. However, a higher magnification showed no morphological distortion of hepatocytes in any case. The degree of inflammatory infiltration was significantly reduced for FGC and FGCL treatments with very few lymphocytic cells around the central veins. No sign of fibrosis was observed in any case of curcumin-bound nanoconjugate treatments. Evident from the observations, curcumin complexation significantly mitigated the inflammatory response in hepatic tissue.

cromatic nuclei with irregular nuclear membranes, whereas the cytoplasm was scanty. Laser-only treatment showed a similar morphology to that of the no-treatment group, depicting discrete necrotic foci with nuclear disintegration. Apoptotic bodies with highly condensed pyknotic, ink-dot-like nuclei and an intensely eosinophilic cytoplasm, as marked by black dashed circles, were detected in all nanoconjugate-treated groups in the presence and absence of a laser. Patches of necrotic regions were also detected in these treatment groups, marked with yellow arrows. Histological analysis indicates both FdU-hairpin-GNR and FdU-hairpin-GNR:curcumin could induce apoptosis to some extent with and without laser irradiation. The observations were further confirmed by immunohistochemical staining.

Figure 5. Histological investigation of tumor sections from different treatment groups. Tumor tissues were processed, sectioned, and stained with hematoxylin–eosin (H&E). The nuclei were stained with the dark purple color of hematoxylin. Necrotic tumor foci (marked with yellow arrows) were homogeneously pink with dark purple dotted structures of degraded nuclei. All of the nanoconjugate treatment groups showed the presence of apoptotic bodies with a bright eosinophilic cytoplasm and condensed nucleus, marked with black dashed circles. The magnification is 400× with a scale bar of 50 μm.

Table 1. Tabular Representation of Hematological Parameters of Different Treatment Groups

| group   | Hb (g/dL) | RBC (10⁶/mm³) | WBC (10³/mm³) | neutrophil (%) | lymphocyte (%) |
|---------|-----------|---------------|---------------|----------------|----------------|
| NT      | 13.5 ± 0.1| 6.8 ± 0.06    | 14.2 ± 0.17   | 50.55 ± 0.8    | 41.4 ± 0.93    |
| L       | 10.2 ± 0.1| 5.4 ± 0.06    | 12.5 ± 0.27   | 50.4 ± 1.06    | 42.2 ± 0.63    |
| FG      | 12.5 ± 0.13| 5.4 ± 0.17    | 11.9 ± 0.13   | 55.4 ± 0.83    | 40.3 ± 0.83    |
| FGL     | 9.6 ± 0.06| 5.3 ± 0.06    | 12.4 ± 0.13   | 52.4 ± 0.77    | 42.8 ± 0.38    |
| FGC     | 12.9 ± 0.06| 6.4 ± 0.17    | 13.8 ± 0.13   | 48.4 ± 0.63    | 48.3 ± 0.73    |
| FGCL    | 12.4 ± 0.1 | 5.7 ± 0.06    | 13.7 ± 0.2    | 45.3 ± 0.46    | 46.45 ± 0.7    |

"Results are expressed as means ± SEM, n = 6, in all cases. Statistical analysis was done using a one-way ANOVA test by Tukey’s multiple comparison method; p < 0.05 was considered statistically significant. Laser irradiation reduced the hemoglobin count in both laser-only (L) and FdU-hairpin-GNR + laser (FGL) groups. Curcumin complexation improved the hemoglobin levels both in the presence and absence of laser irradiation (FGC and FGCL). Changes of the RBC count followed similar pattern to hemoglobin. The WBC count also increased in the presence of curcumin both with and without a laser, compared to FdU-hairpin-GNR and FdU-hairpin-GNR + laser. The neutrophil percent was higher in the FdU-hairpin-GNR treated group and its laser combination, compared to the no-treatment and laser-only group; the percent of neutrophils further decreased in the case of curcumin complexation. The reverse pattern was observed for the lymphocyte percentage."
Figure 7 exhibits H&E-stained lung tissue sections of all studied groups with 400× magnifications (Figure S8, Supporting Information, 100× magnification covering a larger area). The NT group showed a typical alveolar structure with normal thickness of the septa and bronchial walls. The bronchial lumen is designated with the letter “B” in the image. The alveolar septa slightly thickened in the L treatment group. In the FG treatment group, the alveolar septa were severely thickened due to infiltration of inflammatory cells along with erythrocyte deposition, resulting in remarkable shrinkage of alveolar space. Congestion of alveolar space might lead to impaired gas exchange and pulmonary dysfunction, resulting from GNR accumulation. Laser irradiation did not change the conditions significantly; peribronchial thickening and congestion of bronchial space with erythrocytic deposition (Figure S8, Supporting Information) were observed. The degree of inflammation was significantly reduced in both of the curcumin-bound nanoconjugate-treated groups (FGC and FGCL) with little thickening of alveolar septa. Magnification of 400× with a 50 μm scale bar.

Figure 7. Histopathological investigation of H&E-stained lung tissue sections. The bronchial lumen is marked with “B” in all relevant images. The no-treatment group (NT) showed histological features of normal lung tissue with thin alveolar septa and regular thickness of the bronchial wall. The laser-only group (L) showed more thickening of the perivascular space as compared to NT. FdU-hairpin-GNR-treated groups in the presence and absence of a laser (FGL and FG) showed severe interstitial inflammatory cell infiltration with alveolar wall thickening and erythrocyte deposition in the alveolar space. FdU-hairpin-GNR + laser also showed a similar inflammatory response. Inflammatory infiltration was significantly reduced in both of the curcumin-bound nanoconjugate-treated groups (FGC and FGCL) with little thickening of alveolar septa. Magnification of 400× and a scale bar of 50 μm.

Figure 8. Histopathological investigation of H&E-stained splenic tissues. The no-treatment group (NT) showed normal morphological features with distinct white and red pulps and a limited number of megakaryocytes. The laser-only group (L) showed similar morphology to the no-treatment group. An increase in the volume of white pulp along with numerous megakaryocytes (enlarged in the insets) was observed in the case of FdU-hairpin-GNR with and without a laser (FGL and FG) as well as curcumin-bound nanoconjugates with and without a laser (FGCL and FGC). In all images, the white pulp is marked as “WP”, red pulp as “RP”, and fibrous tissue as “F”. Magnification of 100× and a scale bar of 50 μm.

The red pulps of the spleen are rich in cords of Billroth and splenic sinusoids, whereas white pulps are composed of lymphocytes. The increase in the volume of white pulp suggests greater infiltration of lymphocytes as a chronic inflammatory or immune response due to nanoparticle accumulation. A sign of fibrosis in the red pulp regions also supports the response to inflammation in spleen. An increase in the number of splenic megakaryocytes signifies hematopoietic stress. Histopathological investigation of H&E-stained splenic tissues in the Supporting Information show H&E-stained sections of myocardial and renal tissues, respectively. None of the experimental animals demonstrated any morphological abnormality in myocardial or renal tissues.

Immunohistochemical Analysis. Immunohistochemical analysis was done against the active caspase 3 protein to detect apoptotic cells in tumor and vital organs such as the liver, spleen, kidney, heart, and lung. Figure 9 depicts the microscopic images of immunohistochemically stained tumor tissues along with the graphical representation of the apoptotic index. NT and L groups showed minimal presence of cleaved caspase 3 positive cells with percentages of 1.48 and 2.14, respectively. FG treatment with and without a laser showed increased caspase 3+ cells, 5.37 and 5.23%, respectively. The maximum number of apoptotic cells was found in the FGC treatment with 12.98% active caspase 3 expressing cells, while a combination with a laser showed a similar pattern of apoptosis with a value of 10.52%. These increased apoptotic cell percentages for the curcumin complex are consistent with increased anti-tumor activity for these treatment groups (Figure 3). A similar magnitude of apoptosis in the presence as well as absence of a laser suggests no significant photothermal effect, possibly because of very little GNR accumulation in tumor tissue.
METHODS

Gold Nanorod Functionalization and Curcumin Complexation. Gold nanorod synthesis and surface functionalization were performed following the previously published work of Das et al.21 The synthesized GNRs had an average length of 30.0(±5) nm and diameter of 10.0(±2) nm, including absorbance maxima in the range of 790–810 nm. For GNR functionalization, we used a thiolated DNA hairpin with 10 adenine bases at the 3′ end followed by a CGAAG loop and 10 FdU bases at the 5′ end (Figure S1, Supporting Information). FdU containing a thiolated DNA hairpin was purchased from Sigma-Aldrich. The thiolated DNA hairpin was conjugated to gold nanorods via a disulfide bond. The resulting GNR-hairpin conjugates were purified by gel chromatography using Sephadex G-25. The CTAB-
coated GNR suspension was washed to remove excess CTAB. DNA stock solutions were prepared in HPLC-grade water at a concentration of 100 μM. The monomeric DNA hairpin was prepared by heating DNA solutions at 85 °C for 5 min followed by snap-cooling in ice. Thiolated DNA molecules were conjugated on the GNR surface by adding ice-cold DNA solutions in the pre-chilled GNR suspensions. Unattached DNA molecules were removed by centrifuging the conjugation mixture at 4 °C for 1 h at 10,000g; nanoconjugates were collected in a pellet, and free DNA molecules were separated in the supernatant. The final FdU-hairpin-functionalized GNR pellet was resuspended in an equal volume of HPLC-grade water.

For curcumin complexation, a 100 μM stock was prepared by dissolving curcumin powder in absolute ethanol (99% v/v), while mixing on a magnetic stirrer overnight at room temperature (25–30 °C), protected from light. The calculated volume of curcumin was first taken in a microcentrifuge tube, and the FdU-hairpin-GNR conjugate was added then mixed thoroughly to make a final DNA:curcumin molar ratio of ~1:1 and final ethanol concentration of 25% (v/v). The color of the suspension turned yellowish brown from reddish pink. The complexation mixture was then incubated at room temperature (25–30 °C) for 30 min followed by 30 min at 4 °C. The entire process was done protected from light. Following incubation, the reaction mixture was centrifuged at 10,000g for 1 h at 4 °C to remove unbound curcumin. The supernatant was discarded, and the pellet was re-suspended in an equal volume of HPLC-grade water. Curcumin complexation turned the color of the GNR conjugate from reddish pink to yellowish brown (Figure S2a, Supporting Information). DNA attachment and curcumin conjugate from reddish pink to yellowish brown (Figure S2a, Supporting Information). DNA attachment and curcumin complexation were further confirmed by UV–visible and fluorescence spectroscopy.

Spectroscopic Characterization of the Conjugates. UV–visible spectra were acquired under ambient conditions using a TECAN infinite M 200 PRO spectrophotometer. All conjugate suspensions were diluted three times with HPLC-grade water and scanned at a rate of 1 nm/s over the range of 230–1000 nm using a quartz cuvette of 1 cm in path length. For fluorescence spectroscopy, FdU-hairpin-GNR conjugates with and without curcumin complexation were scanned in a CARY 100 fluorescence spectrophotometer. Samples were excited at 425 nm, and emission spectra were recorded for the region of 450 to 650 nm at a scan rate 1 nm/s. Dynamic light scattering spectroscopy of the GNR conjugates was performed using a Zetasizer (Nano S ZEN -1600, Malvern) instrument. One milliliter of the aqueous suspension of each sample was placed in a clear disposable cuvette, and the hydrodynamic diameter was measured at 25 °C using an incident light of 632.8 nm with a detection angle of 173° and the attenuator setting at 7.

Microscopic Characterization of the Conjugates. Any change in the dispersion of GNR conjugates due to curcumin complexation was investigated by transmission electron microscopy. Conjugate suspensions were dropped on 300 mesh carbon-coated copper grids and incubated for 30 min at room temperature (25–30 °C). Excess liquid was removed and air-dried under similar conditions. Samples were visualized with 200 kV of accelerating voltage at ambient temperature using a TECNAI TF 200 transmission electron microscope.

Evaluation of Photothermal Effect in Vitro. The heat emitted by GNR conjugates upon NIR irradiation was evaluated by measuring the change in temperature of their aqueous suspensions with a mercury thermometer. One milliliter of each sample was placed in sealed NIR-transparent glass cuvettes equipped with a thermometer. Aqueous suspensions of varying concentrations, 150, 300, and 600 μg/mL, were then irradiated by an 808 nm laser beam using a CNI-MDL-III 808 (FC) laser system. Samples were irradiated with power levels of 1.0, 1.5, and 2.0 W for 30, 60, or 120 s. The initial temperature of each solution was recorded prior to irradiation, and the maximum temperature was recorded post irradiation. The net temperature increase was estimated by subtracting the heating of pure water in similar conditions.

Development of Tumors in Mouse Models and Evaluation of Anti-cancer Effects. All animal experiments were performed under a protocol approved by the institutional animal ethics committee of the Chittaranjan National Cancer Institute (CNCI), Kolkata. The tumor model was developed by subcutaneously injecting 1 × 107 Ehrlich ascites carcinoma (EAC) cells in the neck region of six week old female Swiss Albino mice. EAC cells were gifted by Dr. Subhadip Hajra (CNCI, Kolkata). Animals were used for the experimental procedure ~14 days following inoculation of the tumor cells when the tumor volume reached ~100 mm3. A total of 36 mice were divided in 6 groups randomly, according to their treatment procedure: (1) NT (no-treatment group), (2) L (laser-only-treated group), (3) FG (FdU-hairpin-GNR-treated group), (4) FGL (FdU-hairpin-GNR and laser-treated group), (5) FGCL (FdU-hairpin-GNR:curcumin-treated group), and (6) FGCL (FdU-hairpin-GNR:curcumin and laser-treated group). Each of the 24 mice from FG, FGL, FG, and FGCL groups was injected with 80 μL of the corresponding conjugate suspension (~150 μg/mL) via their tail veins once a week for four weeks. The total injected dose of gold was ~0.05 mg/animal (~1.6 mg/kg body weight). In the case of the animals from NT and L groups, 80 μL of sterile water was injected in similar conditions. After 4 h of injection, mice from L, FGL, and FGCL groups were irradiated using a CNI-MDL-III-808 (FC) laser with continuous exposure for 90 s at a power level of 1.5 W/cm2. Animals were anesthetized for the laser treatment procedure. Anesthesia was administered by intraperitoneal injection of ketamine HCl (80 mg/kg) along with xylazine (10 mg/kg). The tumor size was measured once a week for 6 weeks following treatment initiation using a Mitutoyo Absolute AOS Digimatic digital caliper. The tumor volume was calculated as V = (tumor length × tumor width2)/2. The relative tumor volume was calculated as V/V0 (V0 is the tumor volume when the treatment was initiated). At the end of 6 weeks, the blood samples were collected from each animal by retro-orbital bleeding. Then, the animals were sacrificed and the vital organs such as the liver, kidneys, spleen, heart, and lungs along with the tumor were isolated. All superficial burned patches on tumors were cut off with significant margins to avoid any artifact for histological and immunohistological analysis.

Investigation of the Biodistribution of Au and Blood Biochemistry. Tumor sections along with the liver, kidney, spleen, heart, and lung were isolated from the treated animals and washed with phosphate-buffered saline, PBS, followed by freezing at −20 °C for later use. Frozen tissue samples were thawed at room temperature and weighed. For each 1 g of the sample, 4 mL of nitric acid and 1 mL of 30% H2O2 were added in a closed Teflon vessel followed by incubation at room temperature for 45 min. Tissues were further digested in a CEM MARS Press microwave digestion system at 160 °C and a power level of 400 W, 100% efficiency, with 20 min of ramping and 15 min of holding time. After digestion, each of the
digested tissue samples were evaporated completely to remove the acid and resuspended in 2 mL of 0.4% HCL (v/v). Atomic absorption spectra were taken at 242.2 nm using a graphite tube atomizer (Varian, GTA120) coupled with a Zeeman atomic absorption spectrometer (Varian, AA240Z) and a hollow cathode gold lamp (Agilent Technologies India Pvt. Ltd). Data were analyzed using SeprataA 5.0 and Microsoft Excel 2010 software.

For the liver and kidney function test, blood samples were collected in non-heparinized tubes and centrifuged at 3000 rpm for 10 min followed by separation of serum fractions. Analysis of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activity was done following the 2,4-DNPH method and Kind and King’s assay, respectively. Serum creatinine and urea levels were determined following alkaline picate and diacetetyl monoxime (DAM) assays, respectively. All serum analyses were done using commercial kits as mentioned in the Supporting Information.

Investigation of Hematological Parameters. For the investigation of haematological parameters, blood samples from all animals were collected in tubes containing heparin (20 IU/mL). Sahli’s method was employed to determine the blood hemoglobin level. Hematological parameters including RBC, WBC, and differential WBC counts were performed by standard procedures.

Histological and Immunohistological Analysis. For histological and immunohistological analysis of tumor and vital organs, tissue sections were fixed in 10% formalin. Tissue embedding, sectioning, and mounting on glass slides were done following standard protocols. For histological analysis, tissue sections were stained with hematoxylin and eosin. For immunohistochemical study, sections were deparaffinized and hydrated following the Abcam standard protocol. The sections were pre-treated with a heat-mediated antigen retrieval method in citric acid buffer (pH 6.0) at 70 °C for 20 min. After antigen retrieval, the sections were completely cooled to room temperature while keeping them in the citric acid buffer. Then, the sections were washed with PBS. The areas around the tissue sections were blotted, and a hydrophobic barrier was drawn around each section using a PAP pen (Sigma-Aldrich, cat. no. Z377821-1EA). Blocking was done using a commercially available immunoperoxidase secondary detection system (Millipore, cat no. DAB150). One drop of 20 μg/mL anti-cleaved caspase 3 antibody (Abcam, cat no. ab2302) was added on each of the sections and incubated overnight at 4 °C in a humid chamber. The biotinylated secondary antibody and streptavidin−HRP conjugate system along with the 3,3′-diamino-benzidine (DAB) chromogen were used for detection, utilizing the reagents and protocol provided in the kit, Millipore, cat. no. DAB150. Both H&E-stained and immuno-stained slides were visualized using a bright-field DM 100 microscope (Leica MikrosystemeVertrieb GmbH, Germany). Images were processed using Image J 1.46 software.

Statistical Analysis. Graphical representations of experimental results were prepared using Microsoft Excel 2010 and Graphpad Prism (version 7). All the experimental data sets are expressed as means ± SEM. Data sets were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test using Graphpad Prism (version 7) software. In all cases, p value < 0.05 was considered statistically significant.
preparing this manuscript. All authors have given approval to the final version of the manuscript. All photos were taken by U.D.

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**Notes**

The authors declare no competing financial interest.

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