Identification of Two Novel Small Compounds that Inhibit Liver Cancer Formation in Zebrafish and Analysis of Their Conjugation to Nanodiamonds to Further Reduce Toxicity

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Liver cancer, which is ranked fourth in cancer-related mortality worldwide, lacks effective therapeutic treatments. The development of new targeted therapies for liver cancer is urgently needed. The zebrafish is an excellent preclinical model organism for drug screening. Therefore, in a zebrafish model, hundreds of small molecules are screened, and two compounds (LIB100078 and LIB100144) are identified as the strongest inducers of antiangiogenic effects without side effects. LIB100078 exhibits better antiproliferation ability, and LIB100144 has better antimigration ability, as shown by xenotransplantation assays. Furthermore, LIB100078 and LIB100144 exhibit anti-HCC effects after retro-orbital injection into adult Tg(fabp10a:HBx,Src, p53-)

1. Introduction

Liver cancer is the fourth leading cause of cancer-related death globally,[1,2] and hepatocellular carcinoma (HCC) constitutes 90% of primary liver cancers.[3] Active hepatitis B virus (HBV) and hepatitis C virus (HCV) continue to be the main cause of HCC.[4] The use of the hepatitis B vaccine has decreased the incidence of HCC in Taiwan.[5,6] Other extrinsic influences, including alcohol consumption, aflatoxin, and tobacco use, together with genetic factors, are closely related to HCC and contribute to the high heterogeneity of liver cancer[6]; hence, personalized medicine is needed.

The treatment options for HCC include resection, liver transplantation, percutaneous ethanol injection, radiofrequency ablation, transarterial chemoembolization, radioembolization, and targeted therapies. Sorafenib, a multiple kinase inhibitor, is the first U.S. Food and Drug Administration (FDA)-approved targeted therapy that can significantly extend survival for advanced HCC patients by 3 months.[7] Another multiple kinase inhibitor, regorafenib, has been a second-line therapy for HCC.

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since 2017. Other targeted therapies include inhibitors of the vascular endothelial growth factor (VEGF) receptor, and anti-EGFR monoclonal antibodies, tyrosine kinase inhibitors, and mTOR inhibitors are being developed as anti-HCC treatments. PTK787, a potent inhibitor of the VEGF receptor, showed potential benefits in the treatment of HCC. Other targeted therapies have been developed but have failed in clinical trials, revealing that understanding the underlying pathways to hepatocarcinogenesis and how to integrate these targeted therapies are urgent needs that require focused attention.

Angiogenesis, the progression of new vessel growth from preexisting vessels, is a critical step in tumor progression. Mechanisms that affect antiangiogenesis have been used to treat cancer, even in clinical settings. The molecular mechanisms underlying tumor angiogenesis and embryonic angiogenesis are similar, and all involved in the VEGF/VEGFR signaling pathway. Zebrafish embryos are a perfect tool for studying tumor angiogenesis. During zebrafish embryogenesis, the inter-segmental blood vessels (ISVs) sprout from the dorsal aorta and the cardinal vein between 16 and 48 h post-fertilization (hpf). Embryos of Tg(fli1:EGFP) transgenic fish express green fluorescent GFP in all blood vessels and thus serve as a perfect model for screening drugs with antiangiogenic effects. ISV formation occurs at 22 hpf from the dorsal aorta (DA). In this study, we used small compounds to treat embryos with a 22 hpf and observed the antiangiogenic effect at 50 hpf. In addition, we used Tg(fabp10a:EGFP-mCherry) transgenic fish, which have the fabp10a promoter to drive the expression of the EGFP-mCherry fusion protein in the liver, for hepatotoxicity assays.

Xenotransplantation is a process in which one special-species tissue or cell is transferred to another animal species. In recent years, xenotransplantation has become a good tool to study human cancer. Because of the advantages of the zebrafish model, xenotransplantation of tumor cells either from cell lines or patients into zebrafish has become a good means to study human cancers in vivo. Patient-derived human cancer cells engrafted into zebrafish have enormous potential as the means to further evaluate cancer progression and discover additional drug therapy. Overexpression of Endothelin 1 (EDN1), a common regulator identified by the HBx-induced HCC mouse model, in HEK-293T cells enhances cell proliferation and migration both in vitro and in vivo. In this work, HEK-293T cells overexpressing EDN1 were labeled with CM-DiI and injected into the 2 days post-fertilization (dpf) zebrafish embryonic yolk sac and then treated with small compounds to observe the antiproliferation and antimigration effects of select drugs.

The Hepatitis B virus × antigen (HBx) antigen is the most studied oncogene in HBV research. Previously, we found that overexpression of HBx in a wild-type background causes hyperplasia or dysplasia at 11 months of age, and HBx synergized with a p53 mutant caused more severe HCC. On the other hand, overexpression of src in a wild-type background caused HCC at 9 months of age, and the overexpression of src in p53-mutant background caused earlier onset HCC, at 7 months. Obesity, defined as a body mass index (BMI) score equal to or higher than 30 kg m⁻² in humans, has become a serious public health problem worldwide, especially in developed countries. Obesity is an important risk factor in many diseases, such as metabolic syndrome, type 2 diabetes, and non-alcoholic fatty liver disease (NFLD). Importantly, obesity is also confirmed as an important risk factor for HCC. Zebrafish is an excellent model for diet-induced obesity, and fish share common pathophysiological pathways of obesity with mammals. We created a transgenic zebrafish line, Tg(fabp10a:HBx, src, p53⁻), which was overfed with Artemia starting at 3 months of age and continually for 8 weeks, and this transgenic fish developed HCC at 5 months of age. By using Tg(fabp10a:HBx, Src, p53⁻) triple transgenic fish injected with or feeding on potentially therapeutic compounds, we examined the anti-HCC ability of these compounds.

Nanodiamond (ND) has the advantages of highly biocompatible, fluorescent, and function-based carriers. Recently, ND has attracted increasing attention with great potential to become a biomedical material applicable to various aspects of treatment. ND as delivery agents have been proven to reduce toxicity and enhance chemotherapeutic efficiency for treatment of tumors. ND-doxorubicin potentially prolongs survival in mice bearing tumors and the ND-epirubicin complex reduces cardiac toxicity and is highly effective treatment against hepatic cancers. Using HepG2 in zebrafish embryos and larvae, folic acid-nanoscale gadolinium-porphyrin metal-organic frameworks (FA-NPMOFs) have been shown to have few biotoxic effects, emit bright red fluorescence, and be delivered to the tumor site due to their high affinity for the folate receptors on HCC cells. Nanodiamonds has greatly applied in cancer biology specifically in chemoresistant hepatic cancer stem cells. Nanodiamond can enhance the treatment against chemoresistant tumor was reported. Thus, we conjugated ND to small molecules for improving the antitumor efficacy. This study was initiated by screening small molecular drugs for antitumorogenic effects. After screening a total of 560 small molecular compounds, we selected six compounds with strong antiangiogenic effects without inducing significant side effects in embryos. Then, we selected two compounds, L1B100078 and L1B100144, which had the lowest IC₅₀ values. In a xenotransplantation assay, we found that L1B100078 showed great antiproliferation ability and that L1B100144 had great antimigration ability. We also used Tg(fabp10a:HBx, Src, p53⁻) triple transgenic fish at the 3 month stage that were overfed for 8 weeks to induce early onset HCC to test whether L1B100078 and L1B100144 could prevent HCC formation. We found that L1B100078 has an outstanding ability to prevent HCC formation and that L1B100144 can also prevent HCC formation but to a lesser
exhibit very high IC50 values and were not used subsequently. Using a patient-derived xenograft model (PDX), we verified that LIB100078 and LIB100144 decrease the proliferation of tumors in patients more effectively than sorafenib. We further prove that nanodiamonds can decrease the toxic effects caused by small molecules. With integrated efforts and taking advantage of the zebrafish platform, we can find more effective and safe drugs for anti-HCC treatments.

2. Result

2.1. Screening 560 LIB1O and LIB1F Derivatives for Antiangiogenic Effects

Induction of antiangiogenesis has become a clinical anticancer strategy, and the Tg(fli1:EGFP) zebrafish embryo provides a powerful high-throughput screening of antiangiogenic compounds. Chemical libraries (2500 compounds) were synthesized from 6 core structures and 320 amines (Figure 1A). First, we screened the original 326 compounds. Embryos were incubated with 20 µm compounds for a 24 h period starting at 1 dpf, and the VEGF receptor inhibitor, PTK787, was used as a positive control, while 1% DMSO was the negative control. The formation of intersegmental vessels (ISVs) of the trunk area was analyzed, while 1% DMSO was 64.7% (Figure S4B, Supporting Information). Among the 36 small molecules with strong antiangiogenic effects from 560 LIB1O and LIB1F derivatives, 16 compounds exhibited strong antiangiogenic ability, and 8 derivatives had minor effects (Figure S2B, Supporting Information). In total, we identified 36 small molecules that exhibited strong antiangiogenic effects and 33 small molecules with minor antiangiogenic effects from 560 LIB1O and LIB1F derivatives.

2.2. Determining the Half-Maximal Inhibitory Concentration (IC50) for Six Selected Compounds

Among the 36 small molecules with strong antiangiogenic effects, six candidates (LIB1F0069, LIB1F0191, LIB1F0318, LIB100078, LIB100144, and LIB100193) were selected for titration experiments developed to determine the IC50 values because the best compound candidates showed strong antiangiogenic activity without inducing embryonic toxicity (Figure S3, Supporting Information). A titration experiment was performed three times to obtain the average value and standard deviation and to determine the IC50 value (Figure 2). LIB1F0318 and LIB100193 exhibited very high IC50 values and were not used subsequently. LIB100078 and LIB100144 had the lowest IC50 values and were comparable to the IC50 value of sorafenib (0.53 µM). Therefore, these two compounds were selected to test their ability to inhibit tumor cell proliferation and migration using a xenotransplantation assay and to prevent cancer formation in adult transgenic fish with liver cancer. The exact chemical name and structure of LIB100078 and LIB100144 were shown in Figure S4A, Supporting Information.

2.3. LIB100078 Exhibited Antiproliferation and LIB100144 Exhibited Antimigration Ability in the Xenotransplantation Assay

Before xenotransplantation, the sublethal dose was determined by immersing Tg(fli1:EGFP) embryos into serially diluted compounds for 2 days starting at 3 dpf. The survival rate of LIB100078 at 2.5 µM was 93.8%, which was higher than that of sorafenib (84.2%), while the survival rate of LIB100144 at 0.3125 µM was 64.7% (Figure S4B, Supporting Information). Based on the sublethal dose experiment, the following amounts were used for the xenotransplantation assay: a 1.25 µM concentration was selected for both LIB100078 and sorafenib, and 0.16 µM was chosen for LIB100144.

Xenotransplantation of tumor cells into zebrafish embryos has potential as a model for screening drugs in vivo.[17,28] We optimized the conditions by incubating the embryos with tumor cells in an incubator and gradually increasing the temperature from 28 to 37 °C over 2 days. Overexpression of EDN1 in 293T cells (293T/EDN1) enhanced the migration and proliferation abilities of the cells.[29] CM-Dil-labeled 293T/EDN1 cells were microinjected into the yolk sac of 2 dpf Tg(fli1:EGFP) embryos. Images of embryos carrying fluorescence-labeled tumor cells were taken 1 day post-injection (dpi) and before drug treatment. Different compounds were then applied to those embryos for 2 days. Then, images of embryos were taken 3 dpi, and the inhibition of proliferation and migration of tumor cells was analyzed by comparing measurements taken 3 dpi to those taken 1 dpi. The fluorescence intensity was quantified using ImageJ. Cell proliferation and migration were determined by comparing fluorescent intensity at 3 dpi versus that at 1 dpi and normalized with that of the fluorescent signal 1 dpi.

In DMSO control embryos, tumor cell proliferation and migration were increased in the majority of embryos (Figure 3). Embryos treated with LIB100078 showed decreased tumor cell proliferation (Figure 3B). On the other hand, embryos treated with LIB100078, LIB100144, and sorafenib showed decreased tumor cell migration compared to the DMSO control (Figure 3C). The statistical analysis showed that LIB100078 significantly reduced the percentage of embryos with tumor cells (Figure 3D) and significantly increased the percentage of embryos with decreased tumor cells (Figure 3E). These data suggested that LIB100078 has the ability to reduce tumor cell proliferation, while LIB100144 and sorafenib can prevent tumor cell migration.

2.4. LIB100078 Prevented HCC in Tg(fabp10a:HBx,Src, p53-) Triple Transgenic Fish with Diet-Induced Obesity

To validate whether the small molecules tested indeed have therapeutic effects on HCC formation, adult transgenic zebrafish with
Figure 1. A) Structures of 326 small compounds. A total of 326 nondisclosed small molecules are starting materials comprising two types: 6 core molecules consisting of a pyrimidine structure (LIB1F, LIB1O, LIB1P, LIB1Q, LIB2Q, and LIB1S) and 320 amine structures (LIB0001–LIB0320). The combination of 6 core molecules and 320 amine structures generated 2500 derivatives. B) Measurement of the lengths of intersegmental vessels (ISVs). Illustration of the embryo with region for measuring the length of ISVs in the red box. Representative images with the ISV lengths of 1, 3/4, 1/2, 1/4, and 0 at 48 h post-fertilization (hpf). ISVs, intersegmental vessels. C) Antiangiogenic percentages after LIB1F treatment at four concentrations (5, 10, 15, 20 µm) and 0 µm is 1% DMSO. D) Antiangiogenic percentages after LIB1O treatment at four concentrations (10, 15, 20, 30 µm) and 0 µm is 1% DMSO. E) Antiangiogenic percentages after LIB0307 treatment at four concentrations (10, 15, 20, 30 µm) and 0 µm is 1% DMSO. F) Inhibition of angiogenesis curve for all compounds plotted by total inhibition percentages at different concentrations of drugs and the IC50 of the LIB1F, LIB1O, and LIB0307 compounds. *0.01 < p ≤ 0.05; **0.001 < p ≤ 0.01; and ***p ≤ 0.001.

HCC were subjected to the drugs, and sorafenib was used as a positive control. LIB100078, LIB100144, and sorafenib (57 µg g⁻¹) were retro-orbitally injected into 5-month-old transgenic Tg(fabp10a:HBx, Src, p53) fish overfed to induce obesity such that they developed HCC. After treating the fish with drugs twice a week for 1 month, the liver tissues were obtained for RNA expression analysis by quantitative-polymerase chain reaction (Q-PCR), and histopathology was examined by hematoxylin and eosin (H&E) staining analysis.

In the expression of marker genes, LIB100078, LIB100144, and sorafenib significantly decreased the expression of cell cycle-related genes (ccne1 and cdk1) compared to the DMSO control (Figure S5A, Supporting Information). Furthermore, LIB100078 and LIB100144 treatment decreased the expression of lipogenic
enzymes (agapt and pap); however, the fish treated with sorafenib retained high levels of lipogenic enzyme (Figure S5B, Supporting Information). In terms of lipogenic factors (chrebp and pparγ), LIB1O0078 treatment decreased the expression level compared to sorafenib and LIB1O0144 (Figure S5C, Supporting Information). Regarding histopathological changes, 67% of fish in the DMSO-treated group developed HCC, and 33% had hyperplasia. Sorafenib treatment decreased the percentage of fish with HCC, all of the fish treated with LIB1O0078 had a normal phenotype, and 60% of LIB1O0144-treated fish displayed steatosis and 40% had hyperplasia (Figure S5D, Supporting Information). These results suggested that although sorafenib and LIB1O0144 exhibited some degree of ability to cure HCC, LIB1O0078 dramatically returned hepatocytes back to a normal state.

2.5. Nanodiamond Has Very Low Cytotoxicity

One of the main problems with anticancer drugs is the toxic effect they create. Zebrafish embryos are excellent for embryonic toxicity assays. Serial dilutions of LIB1O0078, LIB1O0144, and sorafenib were used to treat embryos, and mortality and abnormalities were measured daily until 5 dpf (Figure S6, Supporting Information). The results indicated that LIB1O0144 created strong toxic effects in embryos, causing death at 1.7 µm, while embryos treated with LIB1O0078 and sorafenib exhibited deformities at 1.7 µm. Nanodiamonds (NDs) are function-based carriers with the advantages of high biocompatibility and reduced drug toxicity. Therefore, we coated the nanodiamond with the small molecular drugs. First, the Raman spectra for ND particle size before and after an acid wash were analyzed, and the results showed that NDs disperse after acid wash. The Raman spectra revealed a strong narrow diamond peak located at ≈1332 cm⁻¹ (Figure S7, Supporting Information).

Next, embryo toxicity assays were performed by titration with four nanomaterials (TiO₂ and 5 nm NDs, 50 nm NDs, and 100 nm NDs) at various concentrations (0.00001, 0.0001, 0.001, 0.01, and 0.1 mg mL⁻¹) (Figure S8, Supporting Information). The results suggested that smaller NDs were associated with a substantially lower mortality rate compared to TiO₂; among them, the

![Figure 2](image-url)
50 nm NDs had the lowest mortality rate at all concentrations except that of 0.1 mg mL\(^{-1}\) (Figure S8A, Supporting Information). For the morphology, TiO\(_2\) caused pericardial edema in 12–18% of embryos at the lowest doses (0.00001 and 0.001 mg mL\(^{-1}\)) (Figure S8B, Supporting Information). Nevertheless, none of the three ND sizes caused embryonic abnormalities except for 4–6% of embryos with pericardial edema at the highest concentration (0.1 mg mL\(^{-1}\)) (Figure S8C, Supporting Information). The embryonic toxicity assay indicated that 50 nm NDs had the lowest toxicity, and they were selected for conjugation with LIB100078 and LIB100144 in the subsequent experiment.

Figure 3. Comparing the antiproliferation and antimigration ability of LIB100078 and LIB100144 with Sorafenib by a xenotransplantation assay. A) Representative images of embryos before treatment 1 day post-injection (1 dpi) and after treatment (3 dpi) with DMSO, LIB100078, LIB100144, and sorafenib. B) Embryos treated with 1.25 µM LIB100078 can inhibit cell proliferation better than those treated with 0.16 µM LIB100144 and 1.25 µM sorafenib. C) Embryos treated with 16 µM LIB100144 and 1.25 µM sorafenib can inhibit cell migration better than 1.25 µM LIB100078. D) Statistical analysis of the embryos carrying tumor cells revealed increased cell numbers after drug treatment. E) Statistical analysis of the embryos carrying tumor cells revealed decreased cell numbers after drug treatment.
2.6. Coated Nanodiamond Compounds Reduced the Embryonic Toxicity and Hepatotoxicity of LIB100078 and LIB100144

To characterize the morphology of NDs and ND-BSA-drug complexes, scanning electron microscopy (SEM) was performed (Figure 4). The morphology of NDs was not markedly changed after BSA and drug coatings (Figure 4A, B vs C–F). The embryonic toxicity was examined after exposure to LIB100078 and LIB100144 with and without coated nanodiamonds at various concentrations (0.25, 0.5, 1, 2, 4 µm). Nanodiamond coated with compounds significantly reduced the mortality rate (Figure 5A). NDs also reduced the malformation of the embryos, especially at high doses of LIB100078 (Figure 5B) and LIB100144 (Figure 5C).

The hepatotoxicity assay was developed using transgenic zebrafish embryos with liver cell fluorescence. The hepatotoxicity was measured by immunoblotting the Tg(fabp10a:EGFP-mCherry) embryos (expressing green and red fluorescence in the liver cells) for 2 days starting 3 dpf. Liver fluorescence is decreased when drugs exhibit hepatotoxicity. The hepatotoxicity was examined for LIB100078, LIB100144, and sorafenib without and with conjugated nanodiamonds at various concentrations. Nanodiamond coated with compounds significantly reduced hepatotoxicity, especially for the 2 µm concentration of LIB100078 (Figure 6A) and the 0.5 µm concentration of LIB100144 (Figure 6B). However, the nanodiamond coated with sorafenib showed greater hepatotoxicity, especially at 2 µm (Figure 6C). The results suggested that coating NDs reduced the hepatotoxicity caused by LIB100078 and LIB100144.

2.7. Nanodiamond Does Not Significantly Improve Antiproliferation Ability in the Xenotransplantation Assay

We then examined whether the coating of NDs can improve the antiproliferation ability of LIB100078 and LIB100144. Based on the sublethal dose experiment, we chose 1 µm for both LIB100144 and sorafenib administration and 4 µm for use of
LIB100078 in the xenotransplantation assay. To improve the visibility of tumor cells, stable red fluorescence was used for Hep3B-expressing hepatoma cells in the ND coat experiment. Hep3B_LifeactRFP cells were injected into the yolk of 2 dpf AB wild-type embryos, and the embryos were immersed in LIB100144 (1 µm), LIB100078 (4 µm), or sorafenib (1 µm) with or without ND. The fluorescent area and fluorescence intensity of RFP were quantified using MetaXpress. The cell proliferation rate was determined by comparing RFP at 3 dpi with RFP at 1 dpi normalized with fluorescent signal at 1 dpi. Embryos treated with LIB100144, LIB100078, or sorafenib with or without ND all had significantly lower average fluorescence intensity compared to the DMSO control (Figure 7). However, the nanodiamond did not further improve the antiproliferation ability of the treatment compounds, and in the case of sorafenib, coated ND slightly reduced the antiproliferation ability of the drug.

2.8. Oral Gavage of Nanodiamond Coated with Compound Reduced Tumorigenesis in the Adult Zebrafish Hepatocellular Carcinoma Model

We also validated the antitumorigenic effect of NDs coated with compound using a Tg(fabp10a:HBx,Src, p53-) diet-induced obesity HCC model. LIB100144, LIB100078, and sorafenib with and without ND were administered by oral gavage to 5-month-old transgenic fish for 1 month, and liver specimens were collected for molecular and histopathological examination. The histopathological changes of the Tg(fabp10a:HBx,Src, p53-) transgenic fish with different treatments were measured by H&E stain analysis. Instances of steatosis, hyperplasia, dysplasia, and necrosis were decreased in transgenic fish treated with LIB100144, LIB100078, or sorafenib compared to the DMSO control (Figure 8). Interestingly, the NDs coated with compound seem to have a more normal phenotype compared to those without ND. These results suggested that NDs coated with compounds are more effective and could be used with potential drugs in cancer therapy.

3. Discussion

Malignant tumors are always accompanied by angiogenesis to supply oxygen and nutrients for tumor growth. In particular, HCC is one of the most vascular solid tumors; thus, antiangiogenesis mechanisms are potential therapeutic targets. Sorafenib, the first and only FDA-approved multikinase inhibitor for the treatment of advanced HCC, also targets VEGFR or PDGFR. We established a high-throughput drug screening platform using known antiangiogenesis mechanisms and identified two core molecules (LIB1O and LIB1F) that have antiangiogenic effects. We further determined that many of the LIB1O and LIB1F derivatives exhibited a strong ability to induce antiangiogenesis. Subsequently, we identified two molecules (LIB100078 and LIB100144), which had the lowest IC50 of all the compounds tested, that showed antiproliferation and antimigration abilities in a xenotransplantation model. Furthermore, using a zebrafish HCC model, we found that LIB100078 has the strongest anti-tumor cell proliferation and anti-HCC effects. This finding was...
extremely exciting as a novel small molecule that exhibits anti-HCC effects was identified. We also determined that LIB100144 can prevent cancer cell migration. Both novel small molecules have anti-HCC effects in transgenic adult fish with HCC.

In our laboratory, we have also established a xenotransplantation assay to test whether candidate drugs have antiproliferation and antimigration activities. The large breakthrough was incorporated into the gradually increasing temperature from 28 to 37 °C. We also applied automatic high-throughput analysis for the xenotransplantation assay and developed a robust method for measuring proliferation inhibition and migration inhibition in zebrafish models. Upon establishing a 3D imaging methodology in which images of the same embryo are taken at different time points with confocal microscopy, and custom imaging analysis tools were used for quantifying cancer proliferation with accuracy, the understanding of cell survival and migration was further developed.

Previous efforts dedicated to PDX technology development for oncology drug screening have focused on the use of mouse models that are time consuming and expensive. We have collaborated with clinical doctors to establish an efficient and affordable approach for studying PDXs in a zebrafish model for chemosensitivity testing. In a xenotransplantation assay, we found that LIB100078 had great antiproliferation effects and that LIB100144 had great antimigration ability. In future clinical trials, precision medicine using PDXs may provide personalized medicine according to patient need. When tumor cells migrate, we could use a treatment with LIB100144. When the tumor is in situ, we could use a treatment with LIB100078. If a patient’s cancer is advanced, we can use both drugs. We will combine these two drugs in a PDX zebrafish model and compare the results with those from tests of clinically available drugs. Nanodiamond coated with compounds further reduced the toxic effects of these compounds, which might enable the combinatorial administration of both compounds.

Drug toxicity has always been a problem in the clinical treatment of cancer patients. ND-conjugated doxorubicin has been shown to enhance efficacy and safety in recurrent liver cancer. ND-manganese complexes reportedly enhanced MRI imaging in cancer. ND-mediated drug delivery improved the therapeutic outcome in pancreatic ductal adenocarcinoma. In this study, we found that the embryonic toxicity and hepatotoxicity of LIB100144 and LIB100078 can be further ameliorated by use of coated NDs. With HCC transgenic fish as a preclinical model, we further proved that ND coated with drugs has better outcomes than their noncoated counterparts. It is necessary to have a clinical trial to validate the effect of these compounds in human patients.
Our ultimate goal was to use the PDX integrated with genomic profiling to develop personalized medicines for the treatment of liver cancer. Facilitated by such a high-throughput screening platform, heterogeneous cancer patients are expected to be treated with personalized medicine in a timely manner.

5. Experimental Section

Zebrafish Maintenance: Zebrafish were maintained at the Zebrafish Core Facility at the NHRI. The zebrafish, including embryos, larvae, and adult fish, were maintained at 28 °C under continuous flow in the zebrafish core facility with automated control of a 14 h light (8 a.m. to 10 p.m.) and 10 h (10 p.m. to 8 a.m.) dark cycle. All experiments involving zebrafish were approved by the Institution Animal Care and Use Committee (IACUC) of the NHRI (NHRI-IACUC-105098-A).

Transgenic Zebrafish Lines: Three transgenic zebrafish lines used in this study, Tg(fli1:EGFP), Tg(fabp10a:EGFP-mCherry), tps3f(+/−); Tg(fabp10a:HBx-mCherry,myl7:EGFP) cross with tps3f(+/−); and Tg(fabp10a:src,myl7:EGFP), were used to generate the triple transgenic fish denoted as HBx;src(p53f)

Procedure of Diet-Induced Obesity: Tg(fabp10a:HBx, Src, p53−) transgenic zebrafish at 3 months of age were divided into two groups, each containing 15 fishes, and placed in a 2 L tank. The group designated for overfeeding was fed three times per day with fourfold the regular amount of hatched Artemia (~83 mg cysts/fish/day). The control group was fed one time per day with regular amounts of hatched Artemia (~6.9 mg cysts/fish/day). This procedure was sustained for 8 weeks.

Sources of Compounds: All of the compounds, including 6 core structures, 320 amines, 240 LIB1O derivatives, and 320 LIB1F derivatives, were provided by Dr. Hsing-Pang Hsieh of the Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes. Sorafenib was provided by Drs. Wei-Torn Jiaang and Tsu-An Hsu of the Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes.

Embryos Collection: One day prior to fertilization, the male and female adult zebrafish were placed into mating tanks with a divider to prevent them from mating naturally. On the next morning, the divider was removed, water was changed, and light was given to stimulate the mating process. Embryos were collected 1 h later. After collecting embryos, dead and unfertilized eggs were removed, and embryos were transferred into a 60 mm dish with E3 medium (5 mm NaCl, 0.17 mm KCl, 0.33 mm CaCl2, and 0.33 mm MgSO4) and incubated at 28 °C.

Angiogenesis Inhibition Drug Screening Platform: After collecting the embryos at ~24 hpf, the chorion was removed with protease from Strepto- myces griseus (Sigma-Aldrich) (1.5 mg mL−1 for 5 min). Six dechorionated embryos were placed in one well of a 24-well plate supplied with PTU/E3 buffer with 20 μM drug resuspended in 1% DMSO. Approximately 50 hpf embryos were anesthetized with tricaine to avoid movement, and images of the embryos were taken. To take the image, the length of ISVs was focused to investigate the compound effect on the inhibition of angiogenesis. Six compounds (LIB100078, LIB100144, LIB100193, LIB100609, LIB100191, and LIB10018) that had a strong inhibitory effect on angiogenesis were subjected to serial dilutions to determine the half-maximum inhibition concentration (IC50) for angiogenesis.

Preparation of Drug-Coated Nanodiamonds: Aromatic compounds (e.g., sorafenib) can bind albumin.[19,32] Accordingly, ND-drug composites were prepared by coating drug-albumin complexes on nanodiamonds (NDs). Crude NDs (50 nm) were purchased from Kay Diamond Products (Boca Raton, FL, USA). To remove the nondiamond and graphite-containing layers, the NDs were subjected to acid-wash procedures as previously described.[19] The ND samples (0.5 g) were heated in a 3:1 v/v mixture of concentrated H2SO4 and HNO3 at 75 °C for 3 days, in 0.1 M NaOH aqueous solution at 90 °C for 2 h, and in 0.1 M HCl aqueous solution at 90 °C for 2 h. After washing with deionized water and centrifugation, the resulting NDs were coated with 5% bovine serum albumin (BSA, Sigma-Aldrich) in normal saline for 24 h at 4 °C. Drug-ND complexes were then prepared.
Figure 9. QPCR analysis of cell proliferation marker genes (ccne1, cdk1, and cdk2) in Tg(fabp10a:HBx,Src, p53-) triple transgenic fish after diet-induced obesity treated with DMSO, LIB100078, LIB100144, and Sorafenib without and with coated ND. Tg(fabp10a:EGFP-mCherry) treated with DMSO was used as a control. A) Body weight before oral gavage indicated that the fish were of similar weight. B) ccne1 expression fold compared to Tg(fabp10a:EGFP-mCherry) treated with DMSO. C) cdk1 expression fold compared to Tg(fabp10a:EGFP-mCherry) treated with DMSO. D) cdk2 expression fold compared to Tg(fabp10a:EGFP-mCherry) treated with DMSO. *0.01 < p ≤ 0.05; **0.001 < p ≤ 0.01; and ***p ≤ 0.001.

by incubation of BSA-ND complexes with LIB100078, LIB100144, and sorafenib at desired concentrations. The particle size analysis was conducted using the Horiba laser scattering particle size distribution analyzer LA-950 (Horiba Taiwan; Zhubei City, Hsinchu County, Taiwan). Raman spectra were acquired using a Raman spectrometer (Renishaw 1000B, United Kingdom) equipped with a 532-nm wavelength CW laser (DPGL-2100F; Photop Suwtech, China).

Scanning Electron Microscopy: Field emission-scanning electron microscopy (FE-SEM) images were obtained using a S4700 Type-I scanning electron microscope (Hitachi, Tokyo, Japan). To observe the structure of nanodiamond (ND) samples, NDs (50 nm), ND-BSA, and ND-BSA loaded with compounds LIB100078, LIB1000144, and sorafenib (ND-BSA-78, ND-BSA-144, ND-BSA-sora) powders were seeded on BSA-coated coverslips, fixed by 4% paraformaldehyde in 0.1 m phosphate buffer, and then subjected to drying procedures, and platinum coating, using methods modified from previous reports.[34,35] The prepared samples were observed under a S4700 Type-I scanning electron microscope (Hitachi, Tokyo, Japan). Representative data are shown in Figure 4.

Embryonic Toxicity Test: Zebrafish embryos were harvested at 4 hpf and incubated at 28 °C for the duration of the experiments. Forty embryos were placed into each well of 6-well polystyrene tissue culture plates. Five milliliters solution of a different compound was added to each well. Each compound was tested in various concentration. The buffer was renewed every day throughout the experiment. The morphology and survival rate were observed at different time points: 12, 24, 36, 48, 72, 96, and 120 h. The images were captured using an Olympus SZX10 stereo fluorescence microscope coupled with a DP71 digital CCD camera.

Hepatotoxicity Test: Tg(fabp10a:EGFP-mCherry) embryos were used in the hepatotoxicity assay. At 3 dpf, 20 embryos were placed into one well of 6-well plates with 2 mL of E3 solution with resuspended drugs per well. The embryos were kept in the solution until 5 dpf, at which time images were taken: embryos were anesthetized with 0.012% tricaine, and eight individual images from each group were taken at random with a ZEISS AxiosCam MRc camera. Images were taken in two different settings: one with fixed exposure time to capture red fluorescent protein (RFP) intensity below saturation for the intensity measurements and comparisons and the other with sufficient exposure time to show the whole liver region for size measurement. ImageJ software was then used to quantify the intensity of the RFP and the liver size. The average RFP intensity in the liver was calculated and compared with other measurements taken within the same group of the lateral view fry under the same magnification and fixed exposure time.

Survival Test: Tg(fli1:EGFP) embryos were used in the survival assay. At 3 dpf, 20 embryos were placed into one well of 6-well plates with 2 mL of E3 medium supplemented with drugs. The DMSO control and three different drugs, LIB100078, LIB100144, and sorafenib, were serially diluted to determine the survival rate. Two days after exposure, the embryos were counted, and the survival curves were measured.
Cell Culture: The cell line 293T/EDN1 was established by Mr. Chung-Yi Liao. The 293T/EDN1 cells were cultured with CDMEM, which consisted of Dulbecco’s modified eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37 °C and 5% CO₂.

Establishment of the Hep3B LifeAct-RFP Stable Line: The Hep3B LifeAct-RFP stable line was primarily established by Dr. Shu-Chen Liu of the Department of Biomedical Science and Engineering, National Center University. Dr. Shu-Chen Liu performed lentiviral transfection with the rLV-LifelAct-TagRFP lentivectoral vector (ibidi, Germany) and primary screening RFP cells with 0.5 mg·L⁻¹ puromycin (Gibco, USA). Then, fluorescence-activated cell sorting (FACS) was used to isolate RFP cells with a BD Influx cell sorter (Becton Dickinson, USA) in the Cell Sort Core Lab (CSCL) of the NHRI.

Xenotransplantation Assay: Tg(fli1:EGFP) embryos and 293T/EDN1 cells were used in the xenotransplantation assay. Two days post-fertilization, the Tg(fli1:EGFP) embryos were dechorionated with proteinases and anesthetized with 0.016% tricaine (MS-222, Sigma, USA) before microinjection. The 293T/EDN1 cells were washed and resuspended in PBS and labeled with CM-Dil (red fluorescence) (Vybrant; Invitrogen, Carlsbad, CA, USA). 4.6 nL of injection solution, which contained 200 labeled 293T/EDN1 cells, was injected to the middle of the yolk sac of the embryo via a glass capillary using a Nanoject II nanoliter injector (Drummond Scientific). After injection, embryos were washed with PTU/33 and incubated at a gradually increasing temperature, from 28 to 37 °C in 2 days. One dpi, embryos bearing the tumor cells were photographed twice, once to calculate the red fluorescence for the Dil-labeled cell analysis, and the other to observe the green fluorescence to determine the blood vessels. After imaging, the embryos were treated with DMSO, sorafenib, LIB100078, and LIB100144 separately. Then, images were taken using a fluorescence microscope (LEICA DM IRB). At 3 and 5 dpi, more images were taken to observe cell migration and proliferation changes after drug treatment.

Oral Gavage: Sorafenib, LIB100078, and LIB100144 with or without ND were orally fed to Tg(flap10a:HbS, Src, p53) adult fish overfed for 8 weeks starting at 3 months of age were injected with the selected drugs twice a week for a month, and the histopathology and mRNA changes of the hepatocytes were observed. DMSO was used as a negative control, and sorafenib was used as a positive control. LIB100078, LIB100144, and sorafenib, supplemented with phenol red as a color indicator, were each injected at a concentration of 57 µg per gram adult fish body weight. Each fish was injected at a rate of 0.1 mL per 0.1 g of body weight. The fish were anesthetized with 0.016% tricaine to prevent movement and placed in a deep gel plate facing the proper direction for receiving the injection. The injector needle was pointed at the seven o’clock position and at a 45° angle to the fish. The injection solution was contained in a 2 µL syringe, and the injection was made subcutaneously, where they were free to swim before being released back into the tank.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Complementary DNA (cDNA) was synthesized using a high capacity RNA-to-cDNA kit (Applied Biosystems, Waltham, MA, USA). The reverse transcription (RT) reaction mixture contained 10 µL of 2X RT buffer, 1 µL of 20x enzyme mix, and 1 µg of RNA sample to which RNase-free H₂O was added to form a total volume of 20 µL. The thermal cycling program of the reverse transcription reaction in the PCR machine was set as follows: 37 °C for 60 min to start RT reaction; 95 °C for 5 min to inactive enzyme activity; 4 °C for preservation. For long-term storage, the samples were kept in a −20 °C freezer.

Quantitative-Polymerase Chain Reaction (Q-PCR): After cDNA was synthesized, cDNA was diluted to 100x with RNase-free water. 384-well plates were used to plate cDNA in the Q-PCR. The Q-PCR contained the following ingredients: cDNA (diluted with RNase-free water), 3.8 µL; primer mix (2.5 µm forward and 2.5 µm reverse primer), 1.2 µL; and 2X SyBR Green Mix, 5.0 µL. The specific primers used in the qPCR are listed in Table 1. SyBR Green was added last because of its photosensitivity. The reaction mixture had a total volume of 10 µL. After the reaction mixture was added to the 384-well plate, the plate was sealed with an “optical adhesive cover” and bubbles were smoothed out with a “sealing comb.” Next, the plate was placed into the ABI HT-7900 machine to run the Q-PCR program. The thermal cycling program of the Q-PCR in the ABI HT-7900 machine was set as follows: Stage I: 50 °C for 2 min, 95 °C for 5 min, 4 °C for until the end; Stage II: 95 °C for 10 min; Stage III (40 cycles): 95 °C for 15 s, 60 °C for 1 min; and Stage IV: 95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s.

The starting temperature was 60 °C, and the volume for each sample was 10 µL. Raw data were obtained after the PCR cycle was finished, and it was analyzed as follows: After normalization to the internal control, the ratio of expression levels for the experimental and control groups was calculated using the comparative Ct method. The relative expression ratio (fold change) was calculated based on ΔΔCt = (Ct(target) − Ct(Actin))target − (Ct(target) − Ct(Actin))control and fold change = 2−ΔΔCt. All experiments were performed in triplicate, and the mean values of three values were presented. At least three independent samples were used for Q-PCR, and the standard error was calculated and incorporated into the
presented data as medians ± standard error. Differences among variables were assessed by a two-tailed Student's t-test. A \( p < 0.05 \) was considered statistically significant, which is denoted as follows: *0.01 < \( p \leq 0.05 \); **0.001 < \( p \leq 0.01 \); and ***\( p < 0.001 \).

**Hematoxylin and Eosin Staining (H&E Staining):** The slides were brought to room temperature, and the sections were covered with a liquid blocker (PanPen) to prevent incubation solution evaporation. The slides were incubated with 4% PBS-parafomaldehyde (pH 7.2) for 5 min. The slides were placed in a glass chamber and washed under running tap water for \( \approx 1 \) min to remove excess paraformaldehyde. The slides were stained on a tissue before they were stained.

For hematoxylin staining, the slides were incubated with Mayer’s hematoxylin solution for 5 min in the dark to stain the nuclei. The slides were placed in a glass chamber and washed under running tap water for \( \approx 10 \) min. The oxidized hematoxylin (hematein) combined with metallic salt aluminum sulfate and then interacted with the hydroxy group (–OH group) of water to form aluminum hydroxide. The metallic mordant, hematoxylin-metal formulae were fairly acidic; therefore, the nuclei was first stained purple. However, if excess acid existed, then the aluminum hydroxide was unable to form due to the lack of –OH ions. Tap water was considerably more alkaline than alum hematoxylin (pH ≈ 2.6–2.9), and washing the section in tap water neutralized the acid and the free –OH group and changed the color of nuclei blue.

Furthermore, the slides were incubated in 0.5% eosin solution (Merck, Germany) with acetic acid (1:100) for 10 min to stain fibers in red. The slides were placed in a glass chamber and washed with ddH\(_2\)O for 1 min three times to remove excess eosin, and dehydrated successively in 70% EtOH for 60 s, 90% EtOH for 30 s, 70% EtOH for 30 s, 100% EtOH for 30 s, and xylene for 30 s. Finally, the slides were mounted with one to two drops of xylene-based mounting media and covered with cover slides. They were pressed under a heavy weight for 10 min at room temperature and stored at room temperature. The H&E stain procedure was completed by the Pathology Core Lab in NHRI.

**Statistical Analysis:** Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA) using a two-tailed Student’s t-test. For all statistical analyses, a \( p \) value \(<0.05\) was considered to be statistically significant and is shown as follows: *0.01 < \( p \leq 0.05 \); **0.001 < \( p \leq 0.01 \); and ***\( p < 0.001 \).

### Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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### Conflict of Interest

The authors declare no conflict of interest.

### Keywords

antiangiogenesis, antiproliferation, hepatocellular carcinoma, nanodiamonds

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**Table 1. The primer sequences used for Q-PCR analysis.**

| Name       | Sequences                                                                 |
|------------|---------------------------------------------------------------------------|
| ccne1-F    | 5′ TCCGCACAGGTGCTGAACAAGA 3′                                          |
| ccne1-R    | 5′ TTGCTTTTTCCACGAGCCTTG 3′                                           |
| cdk1-F     | 5′ CCCTCGGGGACCCCTAACAAT 3′                                           |
| cdk1-R     | 5′ CGGATGTCGGCCGGGTCTTCG 3′                                           |
| cdk2-F     | 5′ CAGCTTCCTGGGATATTTCCG 3′                                           |
| cdk2-R     | 5′ CCGAGATCTCTGTTGGTGCA 3′                                            |
| chrebpt-F  | 5′ GGAGATGGACTCGCTCTTTG 3′                                            |
| chrebpt-R  | 5′ GGACAGGGCTCAAAAAGTGGCC 3′                                         |
| pparγ-F    | 5′ GTGTTCACGTCACTCGAGAAGAA 3′                                         |
| pparγ-R    | 5′ TGGTTTACCTGGCTCATTCAAC 3′                                         |
| agpat-F    | 5′ TGGGGAAAAGGCAACGTCG 3′                                             |
| agpat-R    | 5′ CTGGCTATTGACTTTTGGG 3′                                              |
| actin-F    | 5′ TCTCATCATGAGTGGCCACGT 3′                                           |
| actin-R    | 5′ CAGACGGAGTATTGCGCTCA 3′                                            |

[1] K. A. McGlynn, J. L. Petrick, W. T. London, Clin. Liver Dis. 2015, 19, 223.
[2] A. Tang, O. Hallouch, V. Chernyak, A. Kamaya, C. B. Sirlin, Abdom. Radiol. 2018, 43, 13.
[3] a) K. J. Lafaro, A. N. Demirjian, T. M. Pawlik, Surg. Oncol. Clin. N. Am. 2015, 24, 1; b) R. Petruzzello, Gastroenterology 2015, 156, 477.
[4] L. Kulik, H. B. El-Serag, Gastroenterology 2019, 156, 477.
[5] M. C. Wallace, D. Preen, G. P. Jeffrey, L. A. Adams, Expert Rev. Gastroenterol. Hepatol. 2015, 9, 765.
[6] a) I. Archambeaud, H. Auleb, P. Nahon, L. Planché, G. Fallot, R. Faroux, J. Gournay, D. Samuel, S. Kury, C. Feray, Liver Int. 2015, 35, 1872; b) L. Li, H. Wang, Cancer Lett. 2016, 379, 191; c) Y. A. Ghouri, I. Mian, J. H. Rowe, J. Cancingol. 2017, 16, 1.
[7] G. L. Deng, S. Zeng, H. Shen, World J. Hepatol. 2015, 7, 787.
[8] A. G. Duffy, T. F. Greten, Nat. Rev. Gastroenterol. Hepatol. 2017, 14, 141.
[9] F. Bronte, G. Bronte, S. Cusenza, E. Fiorentino, C. Rolfo, G. Cicero, E. Bronte, V. Di Marco, A. Firenze, G. Angarano, T. Fontana, A. Russo, Curr. Med. Chem. 2014, 21, 966.
[10] K. Nguyen, K. Jack, W. Sun, Diseases 2015, 4, 1.
[11] a) P. Fons, G. Gueguen-Dorbes, J. P. Herraert, F. Geronimi, J. Tuyaret, D. Dreherique, P. Schaefler, C. Volle-Challier, J. M. Herbert, F. Bonato, J. Cell. Physiol. 2015, 230, 43; b) E. I. Deryugina, J. P. Quigley, Matrix Biol. 2015, 44–46C, 94.
[12] a) K. J. Gotink, H. M. Verheul, Angiogenesis 2010, 13, 1; b) Q. Zhao, Y. Zhang, J. Guo, J. Li, Mol. Med. Rep. 2015, 12, 1363; c) T. Ochiya, K. Takenaga, M. Asagiri, K. Nakano, H. Satoh, T. Watanabe, S. Imajoh-Ohmi, H. Endo, Mol. Ther.–Methods Clin. Dev. 2015, 2, 15008; d) H. M. Chen, C. H. Tsai, W. C. Hung, Oncotarget 2015, 6, 14940.
[13] a) P. Laakkonen, M. Waltari, T. Holopainen, T. Takahashi, B. Pytowski, P. Steiner, D. Hicklin, K. Persaud, J. R. Tomra, L. Witte, K. Alitalo,
Cancer Res. 2007, 67, 593; b) D. Li, X. P. Li, H. X. Wang, Q. Y. Shen, L. Wen, X. J. Qin, Q. L. Jia, H. F. Kung, Y. Peng, Oncol. Rep. 2012, 28, 937.

[14] C. Tobia, G. De Sena, M. Presta, Int. J. Dev. Biol. 2011, 55, 505.

[15] E. Ellertsdottir, A. Lenard, Y. Blum, A. Krudewig, L. Herwig, M. Affolter, H. G. Belting, Dev. Biol. 2010, 341, 56.

[16] M. Konantz, T. B. Balci, U. F. Hartwig, G. Dellaire, M. C. Andre, J. N. Berman, C. Lengerke, Ann. N. Y. Acad. Sci. 2012, 1266, 124.

[17] C. J. Veinotte, G. Dellaire, J. N. Berman, Dis. Models Mech. 2014, 7, 745.

[18] B. Zhang, C. Xuan, Y. Ji, W. Zhang, D. Wang, Fam. Cancer 2015, 14, 487.

[19] J. W. Lu, Y. Hsia, W. Y. Yang, Y. I. Lin, C. C. Li, T. F. Tsai, K. W. Chang, G. S. Shieh, S. F. Tsai, H. D. Wang, C. H. Yuh, Carcinogenesis 2012, 33, 209.

[20] J. W. Lu, C. Y. Liao, W. Y. Yang, Y. M. Lin, S. L. Jin, H. D. Wang, C. H. Yuh, PLoS One 2014, 9, e85318.

[21] J. W. Lu, W. Y. Yang, S. M. Tsai, Y. M. Lin, P. H. Chang, J. R. Chen, H. D. Wang, J. L. Wu, S. L. Jin, C. H. Yuh, PLoS One 2013, 8, e76951.

[22] a) B. Sun, M. Karin, J. Hepatol. 2012, 56, 704; b) K. Ray, Nat. Rev. Gastroenterol. Hepatol. 2013, 10, 442.

[23] T. Oka, Y. Nishimura, L. Zang, M. Hirano, Y. Shimada, Z. Wang, N. Umemoto, J. Kuroyanagi, N. Nishimura, T. Tanaka, BMC Physiol. 2010, 10, 21.

[24] E. K. Chow, X. Q. Zhang, M. Chen, R. Lam, E. Robinson, H. Huang, D. Schaffer, E. Osawa, A. Goga, D. Ho, Sci. Transl. Med. 2011, 3, 73ra21.

[25] Y. Li, Y. Tong, R. Cao, Z. Tian, B. Yang, P. Yang, Int. J. Nanomed. 2014, 9, 1065.

[26] X. Wang, X. C. Low, W. Hou, L. N. Abdullah, T. B. Toh, M. Mohd Abdul Rashid, D. Ho, E. K. Chow, ACS Nano 2014, 8, 12151.

[27] Y. Chen, W. Liu, Y. Shang, P. Cao, J. Cui, Z. Li, X. Yin, Y. Li, Int. J. Nanomed. 2019, 14, 57.

[28] a) P. Cabezas-Sainz, J. Guerra-Varela, M. J. Carreira, J. Mariscal, M. Roel, J. A. Rubiolo, A. A. Sciarra, M. Abal, L. M. Botana, R. Lopez, L. Sanchez, BMC Cancer 2018, 18, 3; b) D. Hill, L. Chen, E. Snaar-Jagalska, B. Chaudhry, F1000Res. 2018, 7, 1682.

[29] X. Zhang, C. Li, Z. Gong, PLoS One 2014, 9, e91874.

[30] W. Hou, T. B. Toh, L. N. Abdullah, T. W. Z. Yvonne, K. J. Lee, I. Guenther, E. K. Chow, Nanomedicine 2017, 13, 783.

[31] V. S. Madamsetty, A. Sharma, M. Toma, S. Samaniego, A. Gallud, E. Wang, K. Pal, D. Mukhopadhyay, B. Fadeel, Nanomedicine 2019, 18, 112.

[32] a) J. H. Shi, J. Chen, J. Wang, Y. Y. Zhu, Q. Wang, Spectrochim. Acta, Part A 2015, 149, 630; b) M. R. Sahyun, Nature 1966, 209, 613.

[33] P. H. Chung, A. Perevedentseva, J. S. Tu, C. C. Chang, L. C. Cheng, Diamond Relat. Mater. 2006, 15, 622.

[34] M. S. Wong, D. S. Sun, H. H. Chang, PLoS One 2010, 5, e10394.

[35] C. L. Cheng, D. S. Sun, W. C. Chu, Y. H. Tseng, H. C. Ho, J. B. Wang, P. H. Chung, J. H. Chen, P. J. Tsai, N. T. Lin, M. S. Yu, H. H. Chang, J. Biomed. Sci. 2009, 16, 7.