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Proteome Interrogation Using Nanoprobes to Identify Targets of a Cancer-killing Molecule

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Abstract

We report a generic approach for identification of target proteins of therapeutic molecules using nanoprobes. Nanoprobes verify the integrity of nanoparticle-bound ligands in live cells and pull down target proteins from the cellular proteome, providing very important information on drug targets and mechanisms of action. As an example, target proteins for α -tubulin and HSP 90 were identified and validated.

Devastating diseases such as central nervous system disorders and cancer are complex diseases that can result from multiple genetic mutations. In the search for new treatments, simple approaches such as single-target protein screenings often fail to yield innovative drugs. In contrast, multi-targeting molecules are often advantageous in the treatment of these complex diseases by affecting multiple pathways^{1,2}. Drug resistance is also less likely to develop with these molecules¹. However, multi-targeting drugs are difficult to discover through rational drug design or single-protein screening approaches. Phenotype screening and optimization are promising methods for developing such therapeutics, provided that targets and mechanisms of action can be elucidated to show the therapeutic and side effects of the drugs and to develop second-generation drugs with a better profile.

Affinity methods, often used in target identification, use a solid support that attaches active compounds to pull down proteins after incubation with cell lysate³⁻⁶. Bound proteins are separated by SDS-PAGE, and then their identities are determined. However, a major drawback of these methods is that it is not possible to confirm whether the target-binding specificity is altered by chemical modification and the linkage to the solid support due to the large size of testing beads.

Nanoparticles with bioactive compounds on the surface can enter live cells to confirm the desired biological activity and targeting specificity of the modified compound and at the same time identify target proteins by interrogating the cellular proteome in cell lysate. To test this hypothesis, we designed and synthesized compound **1** (Figure 1). Compound **1** selectively killed non-small cell lung cancer H460 cells with an EC₅₀ value of 0.9 μ M and was much less toxic to normal human fibroblasts (EC₅₀ > 100 μ M). However, its primary

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Supporting Information **Available:** Details of the synthesis of compound **2**, quantification of ligand contents of GNP-**2**, cell cycle arrest and apoptosis induced by compound **1**, and the experimental procedure are available. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

targets and mechanisms of action are not known⁷. Compound **3** is a structure analogy of compound **1**, but it did not show cytotoxicity to H460 with an EC₅₀ value of 53.7 μ M. GNP-4 derived from non-active compound **3** was used as a control (Figure 1) for target validation.

Previous structure-activity relationship studies showed that rings B and C were stereochemically confined, while ring A was much more tolerant to structural modifications. We modified ring A (Scheme S1) by attaching a flexible and biocompatible linker in compound **2** and **4**. GNP-2 and GNP-4 (Figure 1) were then synthesized *in situ* using a reported method⁸ (See SI for detailed synthesis and characterizations of GNP-2 and GNP-4).

High-resolution transmission electron microscopic analysis (Figure 2a, b) showed that the average diameter of GNP-2 particles was 2.5 nm (Figure 2b). The GNP-linked ligands were analyzed by high-performance liquid chromatography/mass spectroscopy after cleavage with I₂. The chromatogram (Figure 2c) and the associated electrospray ionization mass spectra (Figure 2d) showed that the peak at 6.6 min corresponded to compound **2** (MW 672.25). On average, there were approximately 65 ligands on each GNP (SI, section 4). Since the chemical modifications and the attachment to a GNP might alter the target-binding specificity, it was necessary to test whether GNP-2 could still enter and kill cancer cells. Figure 3a shows that GNP-2 particles could enter cells readily. Although some GNP-2 particles were found in the cytoplasm, most of them were in endosomes. Cytotoxicity results (Figure 3b) showed that GNP-2 exhibited dose-dependent toxicity to H460 cells, but GNP-4 showed much less toxicity. Because GNP-2 particles were mainly trapped in endosomes, as most nanoparticles are, the reduced cytoplasm entry might be responsible for a higher EC₅₀ value than the free compound **1**. However, GNP-2 maintained its target-binding and anti-cancer activities. Although the protein pulled down with this method will eventually be validated by a series of biological studies, this nanoparticle-based pre-qualification assay in live cells provides key guidance at a very early stage.

Proven active in live cells, GNP-2 and GNP-4 were incubated with cancer cell lysate for 1 h before the bound proteins were separated and analyzed by electrophoresis. More than 10 protein bands ranging from 35 to 150 kDa were observed for GNP-2, while fewer proteins for GNP-4. To ensure that only proteins with specific binding to GNP-2 were correctly identified, we also pre-incubated compound **1** with the lysate for 1 h at 4°C and then incubated this cell lysate with GNP-2 for an additional 1 h. Comparing lane 3 with lanes 2 and 4, only two protein bands showed reduced intensities (Figure 4), indicating that they might be the specific target proteins of compound **1**. The identities of these proteins were determined by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry and data analysis using the Mascot search engine (Table 1). Protein identification results with a protein score confidence interval of 100% were accepted. These proteins were heat shock protein 90-beta (β -HSP 90) and tubulin alpha-1C chain. Proteins α -tubulin⁹⁻¹¹ and β -HSP 90^{12,13} were validated therapeutic targets for cancer treatment. Thus, α -tubulin and β -HSP 90 could be considered as the potential targets of compound **1**.

Among target proteins of compound **1**, tubulin is a key anticancer target. Tubulin-binding agents can stabilize (such as paclitaxel) or destabilize (such as colchicine) microtubule formations, block cell cycle progression, and cause apoptosis. To validate tubulin as a target for compound **1**, we first investigated the compound's effect on tubulin polymerization *in vitro* and on the microtubule assembly-disassembly processes in live cells. Tubulin subunits self-assemble to form cylindrical microtubules in a time-dependent manner (Figure 5a). Colchicine, a microtubule depolymerizing agent, inhibited microtubule polymerization (Figure 5a)^{15,16}. Compound **1** also caused microtubule depolymerization in a manner similar to that of colchicine (Figure 5a). To substantiate this finding, compound **1**'s effects on

microtubule organization in live cells were investigated using immunofluorescence microscopy. The microtubule network in cells treated with 1 μ M colchicine (Figure 5c) or compound **1** (Figure 5b) was disrupted completely. To further validate compound **1** as a microtubule-interfering agents, we investigated compound **1**-induced activation of c-Jun NH2-terminal Kinase (JNK). JNK activation is a hallmark event for microtubule-interfering agents, such as paclitaxel, vinblastine, vincristine, docetaxel and nocodazole¹⁷⁻²⁰. The evelate level of p-JNK by compound **1** was detected and compound **1**-induced JNK activation peaked at 12 h (Figure 5e). These results demonstrated that compound **1** inhibited microtubule organization in live cells by binding to tubulin and inhibiting its polymerization.

In cancer cells, HSP 90 is overexpressed to actively assist folding and maturation of oncogenic proteins such as CRAF-1, ERBB2 and also assist AKT phosphorylation. Blocking HSP 90 leads to degradation of these proteins and inhibition of AKT phosphorylation^{21,22,23}. We investigated whether compound **1**, after binding to HSP 90, inhibited its chaperone activity and caused degradation of CRAF-1 and ERBB2 and inhibition of AKT phosphorylation in comparison with a known HSP 90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldana mycin (17-DMAG)¹². 17-DMAG inhibits HSP 90 and induces proteosome-dependent degradation of CRAF-1,ERBB2 and inhibited phosphorylation of AKT (Figure 6, right panels). Similar protein degradation patterns (CRAF-1 and ERBB2) were also observed for compound **1**. It also induced a time-dependent inhibition of AKT phosphorylation. These results revealed that the anticancer mechanism of action of compound **1** was partly through inhibiting HSP 90's function.

After discovering and validating that compound **1** specifically inhibited tubulin and HSP 90, the mechanism of action of this compound was further substantiated by its role in inducing G2/M cell cycle arrest and apoptosis (Figure S5).

In summary, we identified dual targets for compound **1** in this work using nanoprobes by first validating its anti-cancer activity in live cells and then interrogating the proteome in cell lysate. Our findings demonstrated the power of nanotechnology in drug discovery and chemical biology research. Target identification for therapeutic compounds has been a severely underdeveloped area in drug discovery research and the validation of uncertain targets is tedious and expensive. Nanoprobes will likely play a pivotal role in this area.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structure of **1**, its derivative **2** and GNP-2.

Figure 2.

Characterization of GNP-**2**. a) TEM image of GNP-**2**. Scale bar = 10 nm. b) Size distribution of GNP-**2** according to TEM images. The average diameter is 2.5 nm. Structure identification of free ligands cleaved from GNP-**2** by I_2 . c) Chromatogram. The peak at 5.3 min is I_2 , the peak at 6.6 min is compound **2**. d) ESI-MS spectra corresponding to the peak at 6.6 min.

Figure 3.

a) TEM image of GNP-2 cell uptake. H460 cells were treated with 5 μM GNP-2 for 48 h. Scale bar = 2 μm . b) Percentage growth of H460 cells treated with GNP-2 and GNP-4 for 48 h. The concentrations of GNP-2 and GNP-4 were 0 μM , 1 μM , 2.5 μM , 5 μM , and 20 μM . Results represent means $\pm$ SEM in triplicate. * $P < 0.05$ indicates the percentage growth of the group treated with GNP-2 was significantly different from the 1 group treated with GNP-4 under the same conditions.

Figure 4.

Target identification by GNP-2. H460 cell extract (300 μ L) was incubated with or without 5 mM compound **1** for 1 h at 4°C, and then 0.03 μ mol GNP-2 was added and incubated for 1 h at 4°C. As a control, cell extract (300 μ L) was also incubated with GNP-4 and treated in the same way. Proteins bound to GNPs were separated by 10% SDS-PAGE followed by improved Coomassie brilliant blue G-250 staining¹⁴. Protein bands with lower intensities in the lane with compound **1** than in the lane without compound **1** were identified by MALDI-TOF/TOF MS and Mascot analysis (Table 1).

Figure 5.

Validation of tubulin target. DMSO and colchicine were used as negative and positive controls. a) Microtubule polymerization assay. The concentrations of compound **1** and colchicine were 10 μ M and 5 μ M, respectively. Results represent means $\pm$ SEM from two independent experiments. Microtubule immunofluorescence microscopy images of H460 cells incubated with 1 μ M compound **1** (b), 1 μ M colchicine (c), or DMSO (d) for 24 h. Microtubules were labeled by α -tubulin antibody. Scale bar = 25 μ m. e) Western blot analysis for p-JNK and JNK in the H460 cell extract prepared after 0, 12, 24, and 48 h of treatment with 10 μ M compound **1**.

Figure 6.

Validation of HSP 90 as target. Western blot analysis for HSP 90 client proteins CRAF-1, ERBB2 and p-AKT in the H460 cell extract prepared after 0, 24, and 48 h of treatment with 5 μ M compound **1** or 200 nM 17-DMAG, which is a known HSP 90 inhibitor.

Table 1

Protein identification by MALDI-TOF/TOF MS and Mascot analysis.

Band	Protein	Gene	Protein Score C.I.% ^[a]
1	Heat shock protein HSP 90-beta	Hsp90ab1	100
2	Tubulin alpha-1 C chain	TUBA1C	100

^[a]The protein score confidence interval was calculated using the Mascot search engine to assess the match between the experimental data and the database sequence.