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Hypoxia-directed and activated theranostic agent: Imaging and treatment of solid tumor

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ABSTRACT

Hypoxia, a distinguished feature of various solid tumors, has been considered as a key marker for tumor progression. Inadequate vasculature and high interstitial pressures result in relatively poor drug delivery to these tumors. Herein, we developed an antitumor theranostic agent, **4**, which is activated in hypoxic conditions and can be used for the diagnosis and treatment of solid tumors. Compound **4**, bearing biotin, a tumor-targeting unit, and SN38, an anticancer drug, proved to be an effective theranostic agent for solid tumors. SN38 plays a dual role: as an anticancer drug for therapy and as a fluorophore for diagnosis, thus avoids an extra fluorophore and limits cytotoxicity. Compound **4**, activated in the hypoxic environment, showed high therapeutic activity in A549 and HeLa cells and spheroids. *In vivo* imaging of solid tumors confirmed the tumor-specific localization, deep tissue penetration and activation of compound **4**, as well as the production of a strong anticancer effect through the inhibition of tumor growth in a xenograft mouse model validating it as a promising strategy for the treatment of solid tumors.

1. Introduction

Oxygen (O_2) is essential for all cellular processes in aerobic organisms [1]. Healthy cells and tissues need high levels of O_2 to remain oxygenated and to perform their metabolic functions required for survival. Compared to healthy tissues, solid tumors have low levels of O_2 and a hypoxic environment is created inside solid tumors. Hypoxia is a distinguished feature of various solid tumors and has been considered as a key marker for tumor progression [2]. Various drugs, including the hypoxia-activated prodrugs metronidazole, misonidazole, and etanidazole, have been developed to treat solid tumors, but have failed to show significant improvements [3]. Tumor cells in a hypoxic environment are resistant to many antitumor drugs because of a number of reasons: i) inadequate blood supply, which minimizes the delivery of anticancer drugs [4,5]; ii) poor cellular proliferation [6]; iii) poor sensitivity (p53-mediated apoptosis) to anticancer agents; and iv) increased expression of multi drug-resistant genes [7,8]. In addition to a reduction in cytotoxic effects, hypoxic environments also tend to enhance mutation rates [9], upregulate genes involved in tumor invasion [10] and angiogenesis [11], and stimulate metastatic phenotypes in tumors [12]. These factors reduce the efficacy of anticancer drugs and compromise the treatment of tumors.

The available chemotherapy for solid tumors has increased the survival rate, and improved the symptoms of many solid tumors even though not fully curable. The delivery of antitumor drugs to a solid tumor is extremely challenging in terms of overcoming their limited penetration into the deep tumor cells and enhancing antitumor activity in hypoxic tumors [13–16], although hypoxia-specific imaging probes have been developed based on non-invasive techniques including fluorescence [17–19], magnetic resonance imaging (MRI) [20], and positron emission tomography (PET) [21]. Different functionalized units, including nitroaromatic,

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azo-aromatic, and quinone compounds, have been used to induce hypoxia-selective activation [22,23]. Especially, nitroaromatic compounds are commonly used as a substrate for nitroreductase (NRD) in the presence of reduced NADH under hypoxic microenvironments, owing to their high stability under biological conditions [24].

We developed an antitumor theranostic agent, compound **4**, which is activated in hypoxic environments and can be used for the diagnosis and treatment of solid tumors. Compound **4** comprises biotin and the topoisomerase I inhibitor, SN38, which plays a dual role: it functions as an anticancer drug for therapy and as a fluorophore for diagnosis (Scheme S1) [25,26]. The biotin unit of compound **4** aids in selective targeting of cancer cells and solid tumors, sparing healthy cells, owing to its strong binding to avidin receptors. The nitro unit of compound **4** gets activated exclusively in hypoxic environments and remains inactive in all other conditions, thereby causing no side effects in healthy cells. Compound **4** is activated specifically in the presence of nitroreductase in the hypoxic environments of solid tumors where it releases topoisomerase I inhibitor SN38 and strong fluorescence was observed in the hypoxic region of HeLa cells, tumor spheroids, and solid tumor, relative to the normoxic region. Compound **4** showed high therapeutic activity in A549 and HeLa cells and spheroids, which exhibit high expression levels of biotin receptors. Tail vein injection of compound **4** into HeLa-inoculated xenograft mice revealed its specific accumulation in solid tumors, visualized by its strong fluorescence, and its effect on tumor growth.

2. Materials and methods

2.1. Materials and synthetic procedures

DMTr-chloride (Alfa-Aesar), 2,6-Bis(hydroxymethyl)-4-methylphenol (TCI), 4-nitrobenzyl bromide (Aldrich), EDCI (Carbosynth), DMAP (Alfa-Aesar), TEA (Aldrich), DIPEA (Aldrich), 4-nitrophenyl chloroformate (TCI), SN38 (Carbosynth), potassium carbonate (K_2CO_3) (Samchun Chem.), acetone (Samchun chem.), dichloromethane (Samchun chem.) and DMF (Aldrich) were commercially purchased and used as received. The crude samples were purified using silica gel 60 by column chromatography. IonSpec HiResESI mass spectrometer was used to obtain mass spectra and 400-MHz spectrometer (Bruker, Germany) was used for NMR spectra. Hypoxia chambers were obtained from Billups-Rothenberg (Del Mar, CA, USA).

Synthesis of compound 1: 2,6-Bis(hydroxymethyl)-4-methylphenol (1.68 g, 10 mmol) and 4-nitrobenzyl bromide (4.32 g, 20.0 mmol) were dissolved in acetone (100 mL). Then, K_2CO_3 (6.91 g, 50.0 mmol) was added and refluxed overnight. The reaction mixture was cooled to room temperature and filtered. The filtered solution was evaporated to obtain the crude compound. The crude compound was subjected to silica column chromatography using ethyl acetate (30%) in hexane and dried to afford 2.51 g (82.76%) of compound **1** as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.37 (d, J = 6.21 Hz, 2H), 7.73 (d, J = 6.11 Hz, 2H); 7.25 (s, 2H), 5.01 (s, 2H), 4.53 (s, 4H), 2.31 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 152.21, 148.27, 147.51, 136.23, 133.06, 127.53, 127.51, 123.07, 74.02, 58.39, 20.07 ppm. ESI-MS m/z (M + H): calcd 304.263, found 304.265.

Synthesis of compound 2: DMTr-Cl was added proportionally over a period of 20 min to a solution of compound 1 (2.52 g, 8.27 mmol) in pyridine (50 mL). Then, the reaction was continuously stirred for 6 h. The reaction solution was concentrated and extracted in dichloromethane and dried over anhydrous sodium sulphate. The solution was concentrated and subjected to silica column chromatography (ethyl acetate (20%) in hexane) to afford 2.67 g (53.29%) of compound **2** as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08 (d, J = 7.29 Hz, 2H), 7.49 (m, 3H), 7.37 (m, 10H), 6.82 (d, J = 6.79 Hz, 4H), 4.59 (s, 2H), 4.15 (s, 2H), 3.89 (s, 2H), 3.70 (s, 6H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 158.03, 152.20, 147.89, 146.21, 146.09, 135.51, 133.29, 132.09, 131.22, 130.02, 129.39, 128.67, 128.56, 128.49, 125.54, 121.67, 115.02, 86.01, 73.08, 60.01, 53.2, 20.87 ppm. ESI-MS m/z (M + Na): calcd 628.263, found 628.231.

Synthesis of compound 3: EDCI (191.7 mg, 1.0 mmol) was added to the mixture of biotin (244 mg, 1.0 mmol), and DMAP (61 mg, 0.5 mmol) in dry DMF (20 ml) and the reaction mixture was stirred for 20 min. Compound 2 (605 mg, 1.0 mmol) in DMF (5 mL) was subsequently added to the above mixture and reaction was stirred at room temperature for 10 h. After completion, the reaction was diluted with water and ethyl acetate. The ethyl acetate layer was extracted and dried over anhydrous sodium sulphate and concentrated. The crude product was dissolved in acetic acid (90%) in dichloromethane (20 mL) and continuously stirred for 48 h. After completion of the reaction, the solution was neutralized with sodium bicarbonate solution. The crude compound was extracted in dichloromethane and subjected to silica gel column chromatography to afford 351 mg (66.35%) of compound **3** as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, J = 8.00 Hz, 2H), 7.66 (d, J = 7.29 Hz, 2H), 7.19 (s, 1H), 7.13 (s, 1H), 5.6 (s, 1H), 5.43 (s, 1H), 5.13 (s, 4H), 4.67 (s, 2H), 4.46 (m, 1H), 4.13 (s, H), 3.07 (m, 1H), 2.87 (m, 1H), 2,65 (m, 1H), 2.35 (m, 5H), 1.63 (m, 4H), 1.35 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): 173.35, 163.65, 153.09, 147.59, 144.57, 134.73, 134.22, 131.12, 131.12, 131.04, 129.06, 127.90, 123.82, 61.50, 60.40, 60.06, 55.36, 40.46, 33.95, 28.27, 28.07, 24.71, 20.84 ppm. ESI-MS m/z (M + Na^+): calcd 552.177, found 552.178.

Synthesis of compound 4: DIPEA (129 mg, 1 mmol) and 4-nitrophenyl chloroformate (42.6 mg, 0.2 mmol) were added to a solution of compound 3 (105 mg, 0.2 mmol) in 3 mL dichloromethane at 0 °C and reaction was stirred at room temperature for 3 h. The reaction was concentrated under reduced pressure and crude was intermediate dissolved in 3.0 mL anhydrous DMF and chilled to 0 °C. SN38 (39.0 mg, 0.1 mmol) was added, followed by TEA (100 mg, 1 mmol). The reaction mixture was stirred at room temperature for 24 h and then extracted into dichloromethane. The resulting crude was purified silica gel column using MeOH (5.0%) in dichloromethane as the eluent. The compound was dried in vacuo to afford 41.0 mg (37.60% yield) of compound **4** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (m, 3H), 7.76 (d, J = 7.29 Hz, 1H), 7.65 (m, 3H), 7.39 (s, 1H), 7.23 (s, 1H), 7.19 (m, 1H), 5.47 (s, 2H), 5.42 (s, 2H), 5.39 (s, 2H), 5.21 (m, 2H), 5.02 (m, 1H), 4.92 (m, 1H), 4.27 (m, 1H), 3.21 (m, 2H), 2.87 (m, 1H), 2.57 (m, 1H), 2.35 (m, 5H), 2.02 (m, 4H), 1.92 (m, 2H), 1.62 (m, 4H), 1.42 (m, 5H), 0.98 (t, d, J = 5.60 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 173.92, 173.31, 157.58, 153.73, 153.22, 152.12, 150.33, 149.70, 147.62, 147.33, 146.56, 145,38, 44.21, 135.08, 132.20, 132.5, 131.06, 131.01, 129.09, 127.98, 127.89, 127.82, 127.48, 127.29, 124.48, 123.82, 123.79, 118.81, 113.98, 98.25, 75.85, 72.85, 66.23, 65.99, 61.14, 55.43, 49.39, 40.49, 33.87, 31.58, 29.69, 28.38, 28.26, 24.71, 23.13, 20.85, 13.99, 7.84. ESI-MS m/z (M + Na^+): calcd 970.288, found 970.290.

Synthesis of compound 5: DIPEA (127.8 mg, 0.99 mmol) and 4-nitrophenyl chloroformate (159.48 mg, 0.79 mmol) were added to a solution of compound **1** (200 mg, 0.659 mmol) in 5 mL dichloromethane at 0 °C and reaction was stirred at room temperature for 3 h. The reaction was concentrated under reduced pressure and crude was immediately dissolved in 3.0 mL anhydrous DMF and cooled to 0 °C. SN38 (258.4 mg, 0.659 mmol) was added, followed by TEA (133.4 mg, 1.32 mmol). The reaction mixture was stirred at room temperature for 24 h and then extracted into dichloromethane. The resulting crude was purified silica gel column using MeOH (5.0%) in dichloromethane as the eluent. The compound was dried in vacuo to afford 163 mg (34.2% yield) of compound 5. ^{1}H