



# Targeting colorectal cancer cells with single-walled carbon nanotubes conjugated to anticancer agent SN-38 and EGFR antibody



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## ABSTRACT

In this study, single-walled carbon nanotubes (SWNTs) conjugated with antibody C225 were used to achieve targeted therapy against EGFR over-expressed colorectal cancer cells. In addition, the control release of the chemotherapeutic drug, 7-Ethyl-10-hydroxy-camptothecin (SN38), was studied. We used three different colorectal cancer cell lines, HCT116, HT29, and SW620, listed in the order of decreasing expression levels of EGFR. Our results showed that SWNT could use C225 to specifically bind to EGFR-expressed cells. The cellular uptakes of SWNT of EGFR over-expressed cells (HCT116 and HT29) were much higher than that of the negative control (SW620). We, next, demonstrated that receptor-mediated endocytosis was the primary cell entry route for SWNT. As a consequence, abundant amount of SN38 was released and EGFR over-expressed cells were killed. The drug control release process was studied by utilizing human carboxylesterase enzyme (hCE) that would break the bond linking SN38 and SWNT-carrier in cytoplasm. The intracellular SN38 release observed by confocal microscopy showed that SN38 actually dissociated from the SWNT-carrier first. SN38's entry to nucleus was then followed while the SWNT-carrier still remained in the cytoplasm. Overall, all these data suggested that SWNT could be a good carrier for targeting controlled release therapy.

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## 1. Introduction

Since their discovery in 1991 [1], carbon nanotubes have received continuous interest in a wide range of fields [2–6]. Numerous attempts and efforts have been carried out to fully exploit the properties and potential of functionalized carbon nanotubes. Functionalization of carbon nanotubes can be classified as covalent or non-covalent, depending on the intermolecular interaction [7,8]. Non-covalent functionalization is based on the property of the extended  $\pi$ -system ( $p$ -orbital) of the carbon nanotubes sidewall to bind guest molecules via  $\pi$ -stacking interactions [3,9–11]. It has been shown that hydrophobic molecules

such as surfactants can interact with the hydrophobic surface of the carbon nanotubes sidewall [12]. Moreover, functionalized carbon nanotube carriers are efficient multifunctional biological transporters with no obvious toxicities *in vivo* and *in vitro* [3,4,13].

SN38 (7-ethyl-10-hydroxycamptothecin) is a topoisomerase I inhibitor, a chemotherapeutic drug against various types of cancer, such as colorectal, lung, and ovarian cancer [14]. Despite the excellent anticancer potential, SN38 has not been used as an anticancer drug directly in humans due to its poor solubility in any pharmaceutically acceptable media [15]. Therefore, a potent chemotherapeutic prodrug used in humans is Irinotecan (CPT-11), as it can convert to its active metabolite SN38 by carboxylesterase (predominantly in the liver). Although CPT-11 was approved as an anticancer agent by the US Food and Drug Administration (FDA) [16], SN38 has demonstrated a 1000-fold greater cytotoxicity against various cancer cells *in vitro* than CPT-11 [17]. To overcome the poor solubility disadvantage of SN38, an alternate strategy is to use delivery vehicles that can incorporate SN38 by chemical conjugation or physical entrapment, which can effectively deliver SN38 into the cells [18,19].

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Cetuximab (Erbix, C225) is a chimeric (mouse/human) monoclonal antibody, an epidermal growth factor receptor (EGFR) inhibitor, administered by intravenous infusion for the treatment of metastatic colorectal cancer and head and neck cancer [20–22]. In this study, SN38 was covalently attached to pyrene by a carbamate bond, and the pyrene group attached onto the nanotube surface via strong hydrophobic interactions and  $\pi$ -stacking [23]. We used SWNT/py38 with antibody C225 (SWNT25/py38) to achieve targeted therapy of EGFR over-expressing colorectal cancer cells with controlled release of SN38, and the therapeutic mechanisms of SWNT25/py38 were evaluated.

## 2. Materials and method

### 2.1. PEGylation of SWNTs

Raw single-walled carbon nanotubes were purchased from Golden Innovation Business Co., Ltd (Taipei, Taiwan). To oxidative shortening the length and for further synthesis, nanotubes were oxidized by exposure to strong acid solution [24–26]. The resulting SWNT-COOH was dissolved in PBS, and sonicated for 1 h. Then EDC (Carbodiimides) and NHS (*N*-Hydroxysuccinimide) were added to the nanotubes. The resulting black solution was followed by treatment with  $\text{NH}_2$ -PEG- $\text{NH}_2$  (MW = 6000) and 72 h room temperature stirring during which the precipitate dissolved completely. After stirring, the solution was further washed with water and ethanol to remove any excess reagent. Characterizations of SWNT-PEG<sub>6000</sub> was analyzed by  $^1\text{H}$  NMR and FT-IR (Fig. S1–2).

### 2.2. Synthesis of Py38 conjugation

To a solution of 1-pyrenebutyric acid in anhydrous dimethylformamide (DMF), dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were added. The reaction was stirred at room temperature for 6 h under  $\text{N}_2$ . The activated pyrene butanol (Sigma–Aldrich) and SN38 (ScinoPharm, Taiwan) were stirred in anhydrous DMF at room temperature for 24 h. The resulting solution was purified by washing with ethanol. After dialysis for 5 days, the yellow solid named py38 was collected by filtrated. Characterizations of py38 were performed by  $^1\text{H}$  NMR (Fig. S3).

### 2.3. Attachment of Py38 to PEGylated SWNT

SWNT-PEG was loaded with py38 by drop-wise addition of py38 in THF to a rapidly stirred solution of SWNT-PEG in PBS. The solution was placed under a rapid magnetic stirring system overnight until the THF was totally evaporated. The resulting solution was centrifuged using Amicon®100K centrifugal filtration (Millipore) for purification. The dark film was resuspended in water, and then dialysis. After 5 days, the SWNT-PEG/py38 was collected by filtered. Characterizations of SWNT-PEG/py38 was indicated by UV visible absorbance spectra.

### 2.4. Functionalization of SWNT-PEG/py38 with C225

SWNT-PEG/py38 was dissolved in PBS, and sonicated for 1 h. Then EDC and NHS were added. While the solution was being stirred, C225 (MERCK, Germany) solution was added. The reaction was operated under a magnetic stirring system at 4 °C overnight. The excess reagent were removed using Amicon®100K (Millipore) centrifugal filtration. The dark solid was resuspended in PBS and stored at 4 °C for further application.

### 2.5. Western blot

HCT-116, HT-29 and SW620 cells were lysed in RIPA buffer. The cell lysates were centrifuged at 12,000 rpm for 15 min to pellet the cell debris. The cleared lysates (containing 30  $\mu\text{g}$  of protein) were separated on 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking, the membranes were incubated with polyclonal antibody against human EGFR (Santa Cruz Biotechnology) at 4 °C overnight, followed by a second antibody labeled with horseradish peroxidase (HRP), and developed with enhanced chemiluminescence (ECL) reagents (Santa Cruz Biotechnology). The signals on the blots were scanned and analyzed using the UVP imaging system (BioSpectrum® Imaging System). Each value is shown as an arbitrary unit (AU), and is representative of at least two independent experiments.

### 2.6. EGFR expression

The monolayer of cells was washed with PBS and incubated with trypsin-EDTA at 37 °C for 5 min. The cells were collected and resuspended in medium. The cells were counted using a trypan-blue dye exclusion assay. The cell number in the suspension was adjusted, and  $1 \times 10^6$  cells in 100  $\mu\text{l}$  of medium were used for each test. After incubating the samples with PE-conjugated mouse anti-human EGF receptor antibody (BD Pharmingen) for 20 min at 37 °C in a humidified atmosphere

(5%  $\text{CO}_2$ ) incubator, the cells were washed twice with 3 mL of PBS to remove unbound antibody by centrifugation for 5 min. The cell suspensions were analyzed immediately by flow cytometry.

### 2.7. Cellular uptake of SN-38 carried by SWNT-carriers

HCT116, HT29 and SW620 cells were first seeded onto 96-well plates at a density of 5000 cells per well and cultured for 24 h. The cells were then incubated in media containing various treatments at 37 °C in the dark. The treatments included SWNT25/py38 (SN38 500 ng/ml, C225 3–4  $\mu\text{g}$ /ml), SWNT/py38 (SN-38 500 ng/ml) and free SN38 (SN38 500 ng/ml) for different durations (0, 1, 3, 6, 24 and 48 h). After washing three times with PBS, 100  $\mu\text{l}$  of DMSO were added into each well, and the intracellular SN38 fluorescence was determined using a fluorescence plate reader (Spectra Max M2 Multi-Mode Microplate Reader, BioTek instruments). The excitation and emission wavelengths were set at 380 nm and 420 nm, respectively. Cells were lysed and the extracted proteins were quantified using the BCA protein assay. The fluorescence measurements obtained from each well were normalized to the protein contents in the respective sample, and the results expressed as fluorescence/ $\mu\text{g}$  of protein. Three independent flux experiments, each with triplicate measurements, were performed.

### 2.8. Low-temperature incubation and ATP depletion incubation

HCT116, HT29 and SW620 cells were first seeded onto 96-well plates at a density of 5000 cells per well and cultured for 24 h. Following pre-incubation for 30 min at 4 °C, the cells were incubated in media containing SWNT25/py38 (SN38 500 ng/ml, C225 3–4  $\mu\text{g}$ /ml) for 2 h. After washing three times with PBS, 100  $\mu\text{l}$  of DMSO were added to each well. The intracellular SN38 fluorescence was determined using a fluorescence plate reader. The fluorescence measurements obtained from each well were normalized to the protein contents in the respective sample, and the results expressed as fluorescence/ $\mu\text{g}$  of protein.

HCT116, HT29 and SW620 cells were first seeded onto 96-well plates at a density of 5000 cells per well and cultured for 24 h. After pre-incubation in PBS supplemented with 10 mM  $\text{NaN}_3$  and 50 mM 2-deoxy-d-glucose for 30 min at 37 °C, the cells were incubated with a solution containing SWNT25/py38 (SN38 500 ng/ml, C225 3–4  $\mu\text{g}$ /ml) for 2 h. The samples were then prepared and analyzed following the same steps described above for the cellular uptake. All experiments were carried out in triplicate.

### 2.9. Hypertonic incubation and $\text{K}^+$ depletion incubation

The cells were first seeded onto 96-well plates and the cells were preincubated for 30 min with PBS supplemented with 0.45 M sucrose before exposure to SWNT25/py38 (SN38 500 ng/ml, C225 3–4  $\mu\text{g}$ /ml) at 37 °C for 2 h. The samples were then prepared and analyzed as per the steps described above for the cellular uptake. All experiments were carried out in triplicate.

The cells were first seeded onto 96-well plates.  $\text{K}^+$  depletion was achieved by pre-incubation of the cells in a potassium-free buffer that consisted of 20 mM *N*-(2-hydroxyethyl)piperazine-*N*-[2-ethanesulfonic acid] (HEPES), 140 mM NaCl, 1 mM  $\text{CaCl}_2$ , and 1 mg/mL D-glucose for 30 min at 37 °C prior to incubation in SWNT25/py38 solution (SN-38 500 ng/ml, C225 3–4  $\mu\text{g}$ /ml) at 37 °C for 2 h. The samples were then prepared and analyzed following the same steps described above for the cellular uptake. All experiments were carried out in triplicate.

### 2.10. Drug release profile

*In vitro* release of SN38 from the SWNT25/py38 carrier was analyzed using a dialysis-bag diffusion technique at 37 °C [27]. SWNT25/py38 carriers were dispersed in 100  $\mu\text{l}$  of PBS (at pH 8.0, pH 7.4 or pH 5.0), PBS containing 10  $\mu\text{g}$ /ml (0.1 unit) of hCE2, or 1000  $\mu\text{g}$ /ml of cell lysate (HCT116, HT29 or SW620). Each sample was placed in a Slide-A-Lyzer mini-dialysis device (Thermo Fisher Scientific), and the device was immersed in 50 ml of release medium with continuous gentle stirring at 37 °C. At selected time intervals (0, 1, 2, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168 and 192 h), 100  $\mu\text{l}$  aliquots of the aqueous solution were withdrawn from the release medium. The amount of SN38 was estimated by fluorescence with excitation and emission wavelengths of 390 nm and 580 nm. All experiments were carried out in triplicate.

### 2.11. hCE-2 breaking of the ester bond of Py-38

The hCE-2 (0.1 unit, 10  $\mu\text{g}$ /ml) enzyme was incubated with py38 at 37 °C in 50 mM sodium phosphate buffer (pH 5.0). At selected incubation times, the reaction was stopped by mixing 0.5 ml of reaction solution with 2.0 ml of ice-cold methanol and placing the solution on ice. Samples were lyophilized to dryness and reconstituted in 1000  $\mu\text{l}$  of HPLC mobile phase, and the samples were then analyzed by HPLC (Fig. S11).

### 2.12. Statistical methods

The paired *t*-test was used to determine whether the SWNT-carrier data were statistically significantly different from each of the other treatments. Student's *t*-test analysis was used for selected figures, with  $^{**}P < 0.001$  and  $^{*}P < 0.05$ .

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