

Supporting Information

Nanovectorization of TRAIL with single wall carbon nanotubes enhances tumor cell killing

Al Batoul ZAKARIA ^a, Fabien PICAUD ^a, Thibault RATTIER ^b, Marc PUDLO ^a, Lucien SAVIOT ^c, Rémi CHASSAGNON ^c, Jeannine LHERMINIER ^d, Tijani GHARBI ^a, Olivier MICHEAU ^{b#}, Guillaume HERLEM ^{a#}.

^a Laboratoire de Nanomédecine, Imagerie et Thérapeutique, EA 4662, UFR Sciences & Techniques, CHU Jean Minjoz, Université de Franche-Comté, 25030 Besançon cedex, France

^b INSERM, UMR866, Lipides Nutrition Cancer, Facultés de Médecine et de Pharmacie, Université de Bourgogne, 7 Bd Jeanne d'Arc, Dijon, F-21079 France

^c Laboratoire Interdisciplinaire Carnot de Bourgogne (ICB), UMR 6303 CNRS Université de Bourgogne, 9 Av. A. Savary, BP 47 870, F-21078 Dijon Cedex, France

^d Centre de Microscopie INRA/UB, UMR 1347 Agroécologie, AgroSup/INRA/UB, Pôle Interactions Plantes-Microorganismes-ERL CNRS 6300, 17 rue Sully, BP 86510, 21065 Dijon cedex, France.

Experimental

Ligand production, chemical products and antibodies: His-tagged TRAIL was produced and used as described previously.¹ Regarding functionalization experiments, all the chemicals were of analytical grade and used as received from Sigma Aldrich (Saint-Quentin Fallavier, France).

[#] email addresses : guillaume.herlem@univ-fcomte.fr, Tel.: (33)381662067 (G. Herlem), omicheau@u-bourgogne, Tel.:(33)380393468 (O. Micheau).

Ultra-pure water (Milli-Q-Millipore) was used in all experiments. Anti-TRAIL R1 (B-K32), anti-TRAIL R2 (B-K29), anti-TRAIL R3 (B-H47) and anti-TRAIL R4 (B-R36) antibodies were obtained from Diaclone (Besançon, France). Alexa-488 coupled-goat anti-mouse was from molecular Probes (Invitrogen, Cergy-Pontoise, France). The pan-caspase inhibitor (z-VAD-fmk) was purchased from Alexis (Cergy, Paris, France). The kit for Annexin-marking (Annexin V-FITC, 7-AAD, binding buffer (10x) was from Pharmingen (BD Biosciences, California).

Single-Walled Carbon nanotubes functionalization with TRAIL: Single-Walled Carbon nanotubes (SWCNT) obtained from Unidym were produced by gas-phase catalysis (HiPco process) with an individual diameter equal to 1 ± 0.2 nm and an individual length ranging between 100 nm and 1000 nm (SWCNT median length was around 600 nm) as given by Unidym. Purified SWCNTs were suspended in a 3:1 mixture of concentrated sulfuric and nitric acid (96 % and 69 % respectively). Suspension was sonicated in a water bath (Elmasonic model S30H, 50-60 Hz, 280 W) for 4 h, and then vigorously stirred for 12 h. These two steps were repeated once. Temperature was maintained below 35°C. The resulting suspension was then diluted with 300 mL of water. The resulting nanotubes (SWCNT-COOH) were collected through a 0.2 μ m PTFE membrane, and washed with water until reaching neutral pH and dried under vacuum for 2 h.² The effectiveness of the cutting procedure was controlled by transmission electron microscope (TEM) imagery leading to median length of SWCNT equal to 400 nm.

Preparation of PEGylated SWCNT-PSE and functionalization with TRAIL was performed as follows. 1 mg of SWCNT-COOH was mixed with 3 mg of pyrene butyric acid N-hydroxysuccinimide ester (PSE) in DMF (3 mL). This solution was sonicated in a water bath as

above for 20 min and vigorously stirred for 1 h. The resulting SWCNT-PSE nanotubes were collected through a 0.2 μm PTFE membrane, then thoroughly rinsed three times with sodium phosphate buffer PBS (0.01 M; pH 8), and dried. Next, SWCNT-PSE were mixed with sodium phosphate buffer PBS (0.01 M; pH 8) plus 20 mM EDC and 50 mM sulfo-NHS, and incubated for 1 h at room temperature while stirring. Next, 1 mM preparation of branched PEG 2500-NH₂ was added in order to allow dispersion of SWCNT in aqueous solution, and the solution was incubated overnight at room temperature while stirring. Finally, unbound PEG was removed by filtration through a 0.2 μm PTFE membrane, and the PEGylated nanotubes SWCNT-PSE-PEG dispersed again in water.³ Next, 20 mM EDC and 50 mM sulfo-NHS were added to the solution containing SWCNT-PSE-PEG and the mixture was stirred for 1 h. After that, the protein His-TRAIL (2 mg/mL) was added and the sample incubated over night at room temperature while stirring. Unbound TRAIL was removed by filtration through a 0.2 μm PTFE membrane and the resulting SWCNT-PSE-PEG-TRAIL was washed twice in water. Note that all the synthesized SWCNT-PSE-PEG-TRAIL (NPT) samples were analyzed by TEM microscopy.

Estimation of the mean concentration of grafted TRAIL on nanotubes was inferred from the remaining free TRAIL recovered after functionalization, contained in the three washing solutions, after measuring protein content with the DC protein-assay (Bio-Rad, Marnes-la-Coquette, France), a modified Lowry assay, according to the manufacturer's instructions. The degree of SWCNT-PSE functionalization with TRAIL reached 78% with an estimated concentration of 800 $\mu\text{g/mL}$. This concentration was also estimated by statistical analysis based on images processing obtained from the TEM of SWCNT functionalized with TRAIL (Figure 1B). Calculation of the surface covered with TRAIL compared to the whole accessible nanotube surface was calculated assuming a perfect

cylindrical shape for the SWCNT (around 125600 \AA^2). Then, we approximated the surface occupied by a TRAIL molecule as a sphere of radius $R=35 \text{ \AA}$ (3850 \AA^2). The ratio of the two surfaces (SWCNT over TRAIL) was about equal to 33, which represents the maximum number of TRAIL to obtain a full coverage (100%). The statistical analysis of the TEM images led to an average of 26 TRAIL molecules per SWCNTs (± 2). As inferred from this statistical analysis, TRAIL coverage on the SWCNT corresponded to 80% of the TRAIL added in the reaction mixture at the onset of the functionalization, a value corresponding to the Lowry protein assay described above. These two methods were thus in full agreement.

Cell lines & Cell culture: HCT116 Human colon adenocarcinoma cell lines were cultured with high-glucose Dulbecco's modified Eagle's medium (Lonza, Levallois-Perret, France) supplemented with 10% fetal bovine serum (Lonza). The H1703 Human squamous non-small cell lung cancer and hepatocarcinoma HepG2 cell lines were cultured in RPMI medium (Dominique Dutsher, Brumath, France) containing 10% fetal bovine serum. The Human Hepatoblastoma cell line HUH was cultured in Dulbecco's modified Eagle's medium (Lonza) containing 10% fetal bovine albumin. HCT116, H1703 and SK-Hep1 cell lines were purchased from the American type culture collection (ATCC). HUH & HepG2 cell lines are gifts from EFS Bourgogne-Franche-Comté (Besançon, France).

Measurement of cell viability: In 96-well plates, 40×10^3 cells per well were incubated at 37°C for 16 h with 5% CO_2 and increasing concentrations of his-hTRAIL or nanovector (NPT) from (0 to $15 \times 10^3 \text{ ng/mL}$). Cell viability was measured by methylene blue after fixation of cells by PFA (2%). Optical density (OD) was measured at 630 nm by UV-visible spectrophotometer.⁴

Annexin-V staining methodology: Apoptosis was determined by detection of phosphatidylserine externalization after co-labeling with Annexin V-FITC /7-AAD. Analyses were performed on a cytometer (FACS calibur). Apoptosis is displayed as the percentage of cells presenting a positive staining compared to non-treated cells as control. Each experiment was carried out independently at least three times. Briefly, 300×10^3 cells were cultured overnight in complete medium. Next day, cells were pretreated or not with 20 μ M zVAD-fmk for 1h before stimulating cells with His-TRAIL or NPT for 6 h. Then, cells were trypsinated, centrifuged at 1500 rpm for 5 min, washed in PBS (1X), centrifuged at 1500 rpm for 5 min and stained for Annexin-V and 7-AAD, according to the manufacturer's instructions. The acquisition included a minimum of 10 000 cells.

Hoechst analysis: Nuclear fragmentation was assessed via Hoechst staining by determining the percentage of condensed or fragmented nuclei from at least 300 cells per conditions. 5×10^5 cells were cultured overnight in complete medium. Next day, cells were pretreated or not with 20 μ M zVAD-fmk for 1 h before stimulating cells with His-TRAIL or NPT for 8 h. Cells were then trypsinated, centrifuged at 1 500 rpm for 5 min, incubated for 15 min in a cell disaggregation and counting solution (Accumax: Interchim, Montluçon, France)/PBS (V/V). After centrifugation at 1 500 rpm for 5 min, cells were fixed overnight in a 200 μ L solution (PBS (1X) + PFA (1%) + Hoechst (1 mM)) at 4°C in the dark. Nuclear fragmentation was assessed and quantified using an inverted fluorescence microscope (Leica DMIRB) coupled to an Axiocam device. Pictures were taken and saved with AXIO-Vs 40 V 4.8.2.0 Carl Zeiss MicroImagin GmbH software.

Spectroscopic measurements: Raman spectra of SWCNT, SWCNT-COOH and SWCNT-PSE were measured using a RenishawinVia micro-Raman spectrometer. The excitation laser was provided by the 633 nm line of a 20 mW helium-neon laser. A reduced laser power (0.125 mW on the sample) was required to avoid heating or burning the nanotubes. An x50 microscope objective was used and the acquisition time was 10 s. The intensity of all the Raman spectra were normalized to the intensity of the graphite band (G-band) located at $\sim 1590\text{ cm}^{-1}$.

ATR-IR spectra were performed on Spectrum65 PerkinElmer equipped with ATR module using a Ge crystal.

Experimentally, the three most important Raman active vibrational modes giving rise to the G-band (G for graphite), the D-band (Disorder induced band) and the radial breathing modes (RBM) are visible in the Raman spectra (Fig. S1a). From micro Raman spectroscopy, many changes during the functionalization process can be observed such as upshift (2.2 cm^{-1}) and spectrum shape variation contrary to bulk Raman studies.⁵ In a less extent, some useful information can be extracted from ATR-IR spectra Fig. S1b. Comparison between bare and modified SWCNT highlights important differences such as the presence of -OH groups (broad peak between 3000 and 3600 cm^{-1} in the SWCNT-PSE spectra). Contrary to Raman scattering of PSE generating fluorescence, the IR-active signal of PSE is visible and very intense (between 1700 cm^{-1} and 1800 cm^{-1}), and can be related to the weak interaction between PSE and the SWCNT.

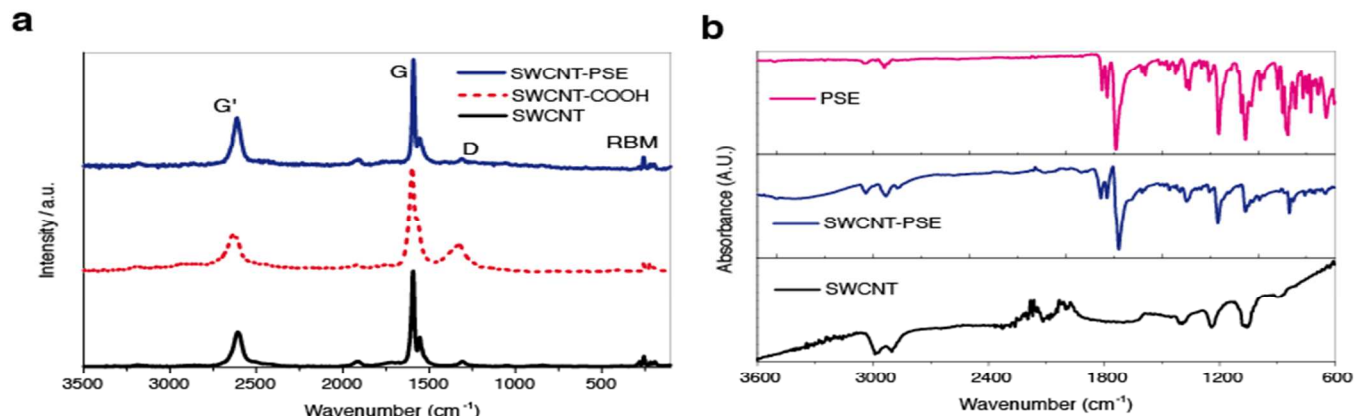


Fig. S1. (a) Raman spectra of SWCNT, SWCNT-COOH and SWCNT-PSE showing the presence of the D, G (G⁺ and G⁻), and G' bands. (b) Experimental IR spectra of SWCNT, SWCNT-PSE and PSE.

Transmission Electronic Microscopy (TEM): The different samples (control carbon nanotubes, functionalized carbon nanotubes with and without grafted TRAIL) deposited on collodion coated carbon grids were freshly prepared on site before observation (diameter 3.05 mm): 10 μ L/grid. A blocking solution (TBS 10 mM, acetylated BSA (BSA-c) 0.5% from AURION, glycine 0.1%, Normal goat serum (NGS) from AURION 10%) was added on each grid for 30 min, followed by 5 washings with the rinsing solution TBS/BG (TBS 10 mM, BSA-c 0.5%, glycine 0.1%). Then a polyclonal anti-histidine anti-rabbit antibody (1/100) (His-probe H-15, Santa Cruz Biotechnology) diluted in TBS was deposited for 1 h. Grids were rinsed 5 times with TBS/BG and incubated with the secondary GAR antibody (a Goat anti-rabbit IgG antibody coupled with gold particles of 1nm diameter for Electronic Microscopy, from AURION (Wageningen, The Netherlands), (1/100) was deposited for 1 hour followed by 5 washings with TBS/BG. Then, a fixing step with a solution of glutaldehyde 2% during 7.5 min was performed and followed by rinsing 5 times with distilled water. Finally, the signal was amplified with silver (AURION R-

GENT SE, Electronic Microscopy) for 21 min in the dark. Grids were next rinsed 5 times with distilled water and dried for 1 h before analysis under the microscope (JEOL JEM-2100F microscope operating at 200 kV and equipped with a ultra-high resolution pole piece achieving a point- to-point resolution of 0.19 nm) operating at 80 kV.

Immunoprecipitations: For DISC analysis, 50×10^6 cells were stimulated with 2 $\mu\text{g/ml}$ of His-hTRAIL (or NPT) in 6 mL of complete medium, for the indicated times at 37°C. Cells were then washed with PBS 4°C (Dominique Dutscher), and lysed in 1 mL lysis buffer containing 1% of detergent (NP40 1% (Nonidet P40, Sigma-Aldrich), 20 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, and 10% of glycerol) in the presence of protease inhibitors (pepstatin A 2.5 mg/mL, aprotinin, 10 mg/mL trypsin inhibitor 2.5 mg/mL leupeptin, 5 mg/mL, Roche, France) for 20 min, then centrifuged for 15 minutes at 13 000 rpm at 4°C. Lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich) for 1 h at 4°C with gentle shaking, and immunoprecipitated at 4°C overnight with G-protein Sepharose beads (Amersham Biosciences, Les Ullis, France) in the presence of 2 μg of anti-Caspase-8 antibody (Santa Cruz C-20). Beads were then washed three times, and immunoprecipitates were eluted in loading buffer (Tris-HCl 63 mM, SDS 2%, phenol red 0.03%, glycerol 10%, DTT 100 mM, pH 6.8), boiled for 5 min and processed for immunoblotting.

Western blotting: Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Bio-Rad) membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.5% Tween 20 and 5% powdered milk. Immunoblots were then incubated with the following specific primary antibodies FLIP NF6

(Adipogen), FADD (BD Transduction Laboratories), Caspase-8 &10 (MBL), DR4, DR5 (Merck Millipore), followed by horseradish peroxidase-conjugated secondary antibody. The membranes were then revealed with ECL (Advansta, WesternBright Quantum, Western blotting detection kit) by the enhanced chemiluminescence method according to the manufacturer's protocol. The chemiluminescence was detected with an imaging system 'Chemi-Doc' (Bio-Rad). Then, the results were finally analyzed with the Image Lab software.

Statistical analysis: Statistical analysis was performed using the non-parametric analysis of variance (ANOVA) with Bonferonni *post hoc* multiple comparison test. P value ***<0.001 was considered significant. NPT results were compared with TRAIL ones. All statistical analyses were performed using Prism 5.0a software (GraphPad Software, San Diego, CA.).

References

- (1) Schneider, P. *Methods in enzymology* **2000**, 322, 325-45.
- (2) Liu, J.; Rinzler, A. G.; Dai, H.; Hafner, J. H.; Bradley, R. K.; Boul, P. J.; Lu, A.; Iverson, T.; Shelimov, K.; Huffman, C. B.; Rodriguez-Macias, F.; Shon, Y. S.; Lee, T. R.; Colbert, D. T.; Smalley, R. E. *Science* **1998**, 280, 1253-56.
- (3) Heister, E.; Neves, V.; Lamprecht, C.; Silva, S. R. P.; Coleya, H. M. C.; McFadden, J. *Carbon* **2012**, 50, 622-32.
- (4) Merino, D.; Lalaoui, N.; Morizot, A.; Schneider, P.; Solary, E.; Micheau, O. *Mol. Cell. Biol.* **2006**, 26, 7046-55.
- (5) Karachevtsev, V. A.; Stepanian, S. G.; Glamazda, A. Y.; Karachevtsev, M. V.; Eremenko, V. V.; Lytvyn, O. S.; Adamowicz, L. *J Phys Chem C* **2011**, 115, 21072-82.