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

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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.

ABSTRACT: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) is a member of the tumor necrosis factor (TNF) superfamily. This type II transmembrane protein is able to bound specifically to cancer cell receptors (i.e. TRAIL-R1 (or DR4) and TRAIL-R2 (or DR5)) and to induce apoptosis without being toxic for healthy cells.

email addresses : guillaume.herlem@univ-fcomte.fr, Tel.: (33)81662067 (G. Herlem), omicheau@u-bourgogne, Tel.:(33)380393468 (O. Micheau).

Since membrane-bound TRAIL induces stronger receptor aggregation and apoptosis than soluble TRAIL, we proposed here to vectorize TRAIL using single walled carbon nanotubes (SWCNTs) to mimic membrane TRAIL.

Owing to their exceptional and revolutional properties, carbon nanotubes, especially SWCNTs, are used in a wide range of physical or, now, medical applications. Indeed due to their high mechanical resistance, their high flexibility and their hydrophobicity, SWCNTs are known to rapidly diffuse in an aqueous medium such as blood, opening the way of development of new drug nanovectors (or nanocarriers).

Our TRAIL-based SWCNTs nanovectors proved to be moreefficient than TRAILalone death receptors in triggering cancer cell killing. These NPTs increased TRAIL pro-apoptotic potential by nearly 20 fold in different Human tumor cell lines including colorectal, non-small cell lung cancer or hepatocarcinomas. We provide thus a proof-of-concept that TRAIL nanovector derivatives based on SWCNT may be useful to future nanomedicine therapies.

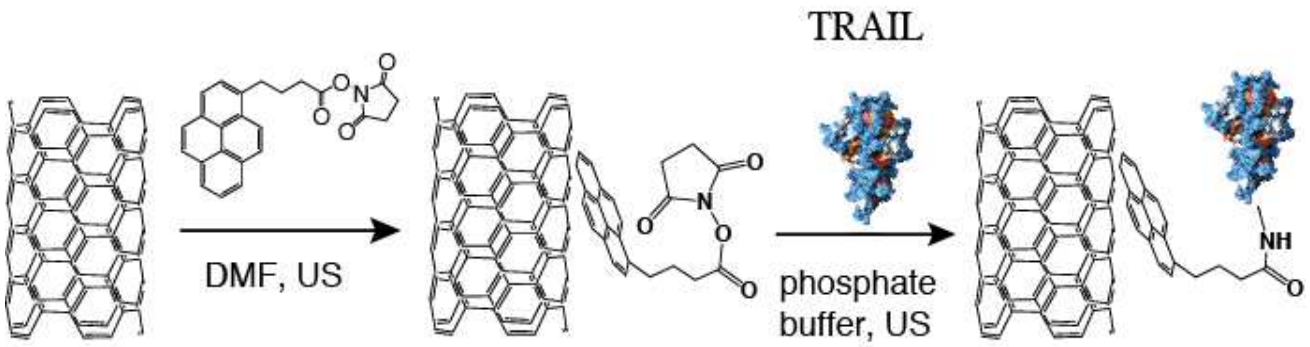
KEYWORDS: carbon nanotubes; nanovector; TRAIL; death receptor; nanomedicine; cancer therapy

In the emerging industry of nanotechnologies, carbon nanotubes (CNTs) can be used for nanovector engineering.[1-5]Due to its antitumor properties without any damage for healthy cells, TRAIL ligand has attracted major interest in oncology. Indeed, itis able to induce cancer cell apoptosis by binding to TRAIL-R1 (DR4) and TRAIL-R2 (DR5) receptors (known as death receptors). But, despite clear evidence of antitumoral activity, clinical trials demonstrated that the use of TRAIL or TRAIL derivatives alone was not sufficient to cure patients.[6,7]

In this context, current therapeutic approaches aim at combining TRAIL with conventional chemotherapy or targeted therapies to circumvent different resistances to TRAIL-induced apoptosis due to the presence of the antagonist receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2),the caspase-8 inhibitor c-FLIPor to a defect in the mitochondrial pathway.[8-10] Alternatively, mutagenesisor multimerization of TRAIL (or TRAIL derivatives)to mimic native membrane-bound TRAIL are explored to increase TRAIL efficacy.[11-13]

Multivalent TRAIL mimetic peptides or nanobodies targeting anti-TRAIL-R2 have recently been developed and have demonstrated superior killing activity than their monovalent counterpart or TRAIL.[13, 14]Herein, we proposed to synthesize novel nanovectors based on SWCNT functionalized with TRAIL via noncovalent 1-pyrenebutanoic acid N-hydrosuccinimid ester (PSE) as shown on Scheme 1.[15]These TRAIL-based nanovectors (noted NPT thereafter) are intended to increase TRAIL valency and to enhance apoptosis induced by both TRAIL-R1 and TRAIL-R2, as compared to soluble TRAIL.

Scheme 1. Illustration of chemical reactions used for TRAIL grafting on SWCNT



Before delivering NPT on cancer cell lines, the mean concentration of grafted TRAIL on nanotubes was estimated by Lowry's protein quantification assay and statistical analysis based on images processing from TEM (see S.I. for details). The degree of SWCNT-PSE functionalization with TRAIL was about 80%, corresponding to a final concentration of 800 µg/mL The resulting functionalized nanotubes were analyzed by TEM for the presence of TRAIL and compared to shortened SWCNT (Figure 1A and 1B).

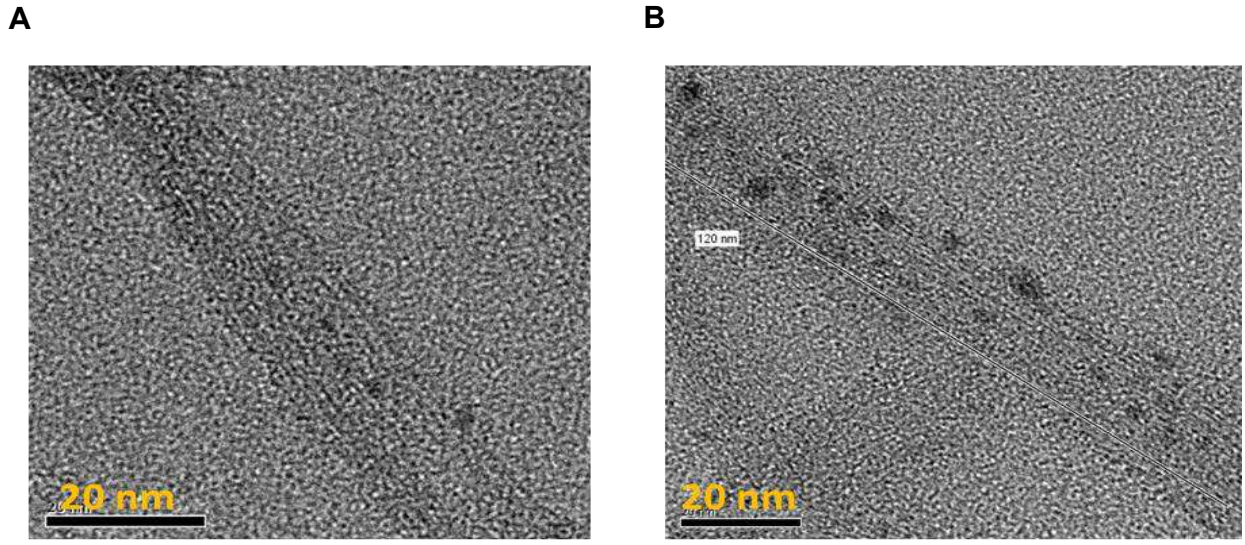


Figure 1. (A) TEM of SWCNT-PSE, and (B) TEM of TRAIL coated SWCNT bundle.

To assess the pro-apoptotic activity of NPT as compared to TRAIL, several tumor cell lines expressing TRAIL-R1 and TRAIL-R2, but little to no TRAIL-R3 or TRAIL-R4 (Figure2A) were seeded in 96-well plates. A progressive increase of theTRAIL (or NPT) concentration wassubmitted to the culture medium. 16 hours after the stimulation, plates were washed and remaining viable cells were stained with methylene blue to measure cell viability. Both NPT and TRAIL induced a dose dependent inhibition of the cell viability of all the hepatocarcinoma cell lines, *i.e.*SK-Hep1, HUH and HepG2, and of the non-small cell lung carcinoma cell line H1703 (Figure 2B).However, as expected, TRAIL-basednanovectors (NPT) displayed better cytotoxic activity than recombinant soluble TRAIL for all tested cancer cell lines.

While 88 ng/mL TRAIL was required to kill 50 % of H1703 cells, the IC50 was reached with only8 ng/mL NPT, demonstrating that NPT action was largely superior than TRAIL one in inducing apoptosis of these cells. NPT was thus 10 fold more potent than soluble TRAIL.

The experiments carried on Hepatocarcinoma cells (SK-HEP1, HepG2 and HUH) showed that NPT was also 10 to 20 fold more efficient than TRAIL to induce cell death. IC50 estimated for TRAIL in these cells ranged from 650, to more than 2600 ng/mL (Figure 2B) while NPT IC50 ranged from 33 to 230 ng/mL (Figure 2B). Remarkably, the gain of function obtained with TRAIL functionalized on SWCNT in HepG2 cells, which expresses the functional antagonist receptor TRAIL-R4 in addition to TRAIL-R1 and TRAIL-R2 was around 20 fold as compared to soluble TRAIL.

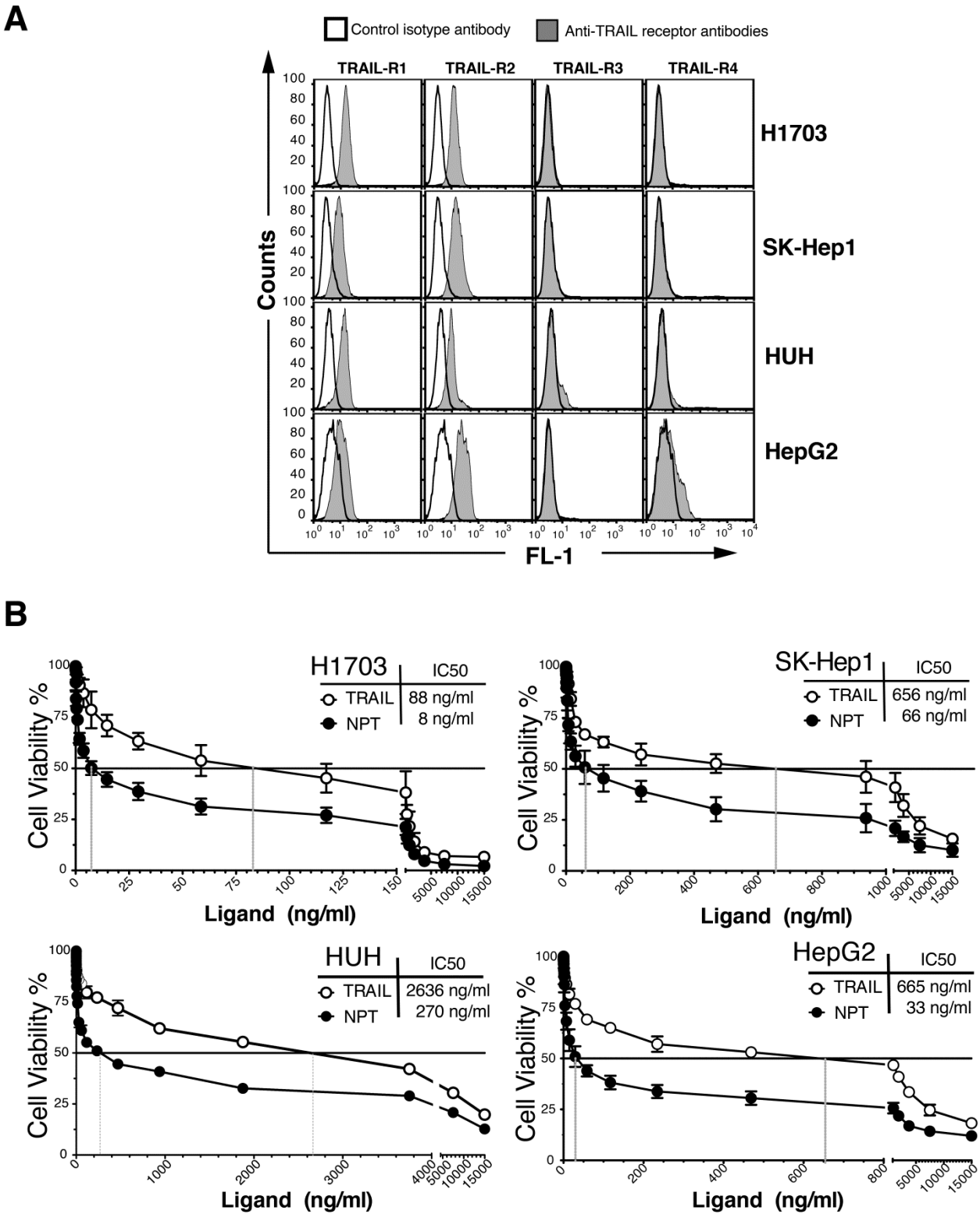


Figure 2. (A) Flow cytometric analysis of TRAIL receptor expression in the non-small lung carcinoma cell line H1703 and in the hepatocarcinoma cell lines, SK-Hep1, HUH and HepG2. The

white histogram represents the isotype control antibody (IgG). The grey histogram corresponds to TRAIL receptor staining. (B) Cell viability in H1703, SK-Hep1, HUH and HepG2 cells after stimulation with increasing concentrations of TRAIL or NPT. Viability was measured by methylene blue staining. Data are the mean ± SD (n=3).

To prove again the better ability of NPT to induce cell death compared to TRAIL alone, apoptosis was assessed in H1703 cells by Hoechst and Annexin-V staining using a fixed concentration of TRAIL and NPT. As shown by fluorescent microscopy for Hoechst staining, both TRAIL and NPT induced nuclear fragmentation, a hallmark of apoptosis (Figure 3A). Inhibition of caspase activation by the pan-caspase inhibitor ZVAD inhibited nuclear fragmentation-induced by TRAIL and NPT, demonstrating that NPT was able to trigger apoptosis via TRAIL-R1 and TRAIL-R2, like TRAIL. Quantification of H1703 undergoing apoptosis upon NPT stimulation clearly demonstrates that NPT is superior to TRAIL (Figure 3B).

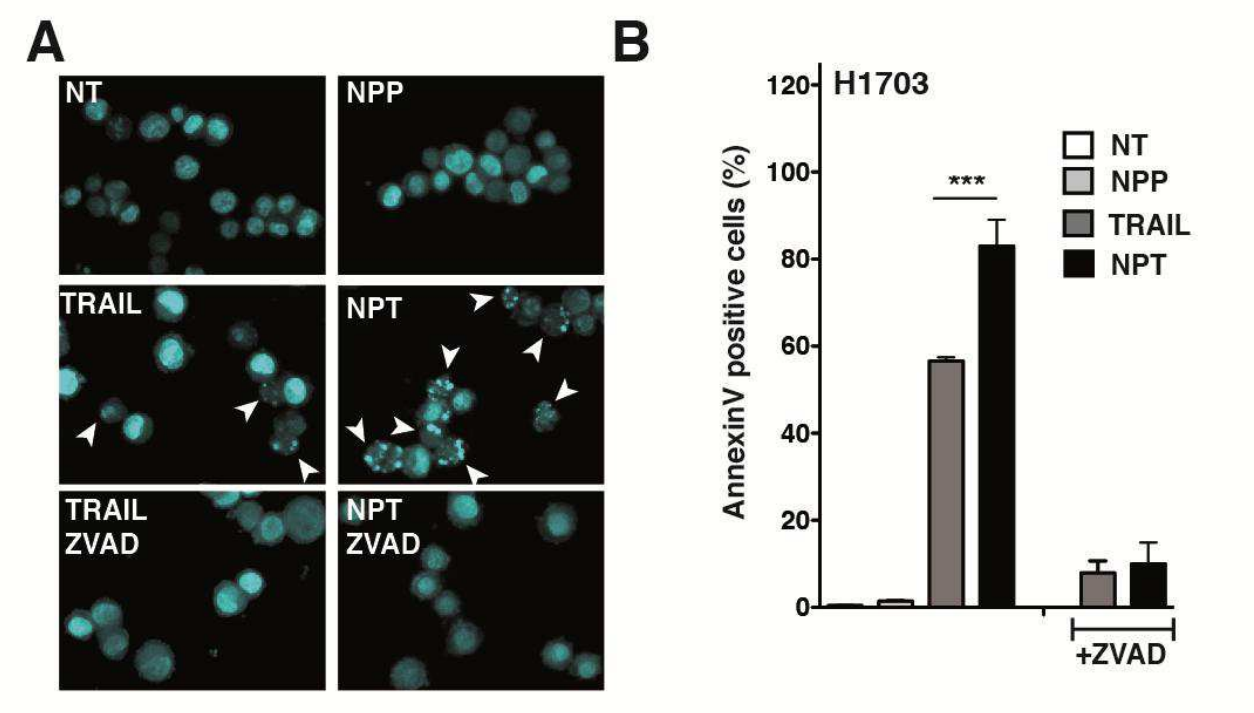


Figure 3. (A) Hoechst staining of H1703 cells after treatment with the same concentrations of NPT and TRAIL (20 ng/mL): Enumeration of apoptotic nuclei (about 300 cells) of H1703-WT cell line.The white arrows show fragmented or condensed nuclei. (B) Percentage of apoptotic cells. Untreated cells (NT) are used as control. Data are the mean ± SD (n=3). Differences between selected groups were compared to non-parametric analysis of variance (ANOVA) with Bonferonni *post hoc* multiple comparison test, ***P < 0.001, compared to TRAIL stimulation alone and NPT in H1703 (WT) cell lines. NT: Non-treated, NPP: Control Carbon nanotubes, ZVAD: Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone).

In order to demonstrate that the gain of function of NPT was only due to TRAIL and not to nanoparticles themselves, we took advantage of a colon carcinoma cell line in which both agonist receptors were knocked-out (paper in preparation). Parental HCT116 cells (HCT116 WT) express TRAIL-R1 and TRAIL-R2 (Figure 4A) and are sensitive to TRAIL-induced cell death (Figure 4B). TRAIL IC50 value in these cells was determined close to 40 ng/mL. Using HCT116 cells (HCT116 DKO) deficient in TRAIL-R1 and TRAIL-R2, TRAIL was unable to trigger apoptosis and thus cell survival was not affected whatsoever the amount of TRAIL. In these cells, NPT was also unable to reduce cell viability (Figure 4B) demonstrating the non-toxicity of the nanoparticles. However in WT cells, as in the other cancer cell lines tested here, NPT was more efficient than TRAIL in reducing cell viability. Its IC50 value was estimated at 3 ng/mL, indicating that NPT was at least 10 fold more efficient than soluble TRAIL to kill HCT116 WT cells, while it remained unable to trigger apoptosis in TRAIL receptor deficient HCT116 cells (DKO), like TRAIL itself.

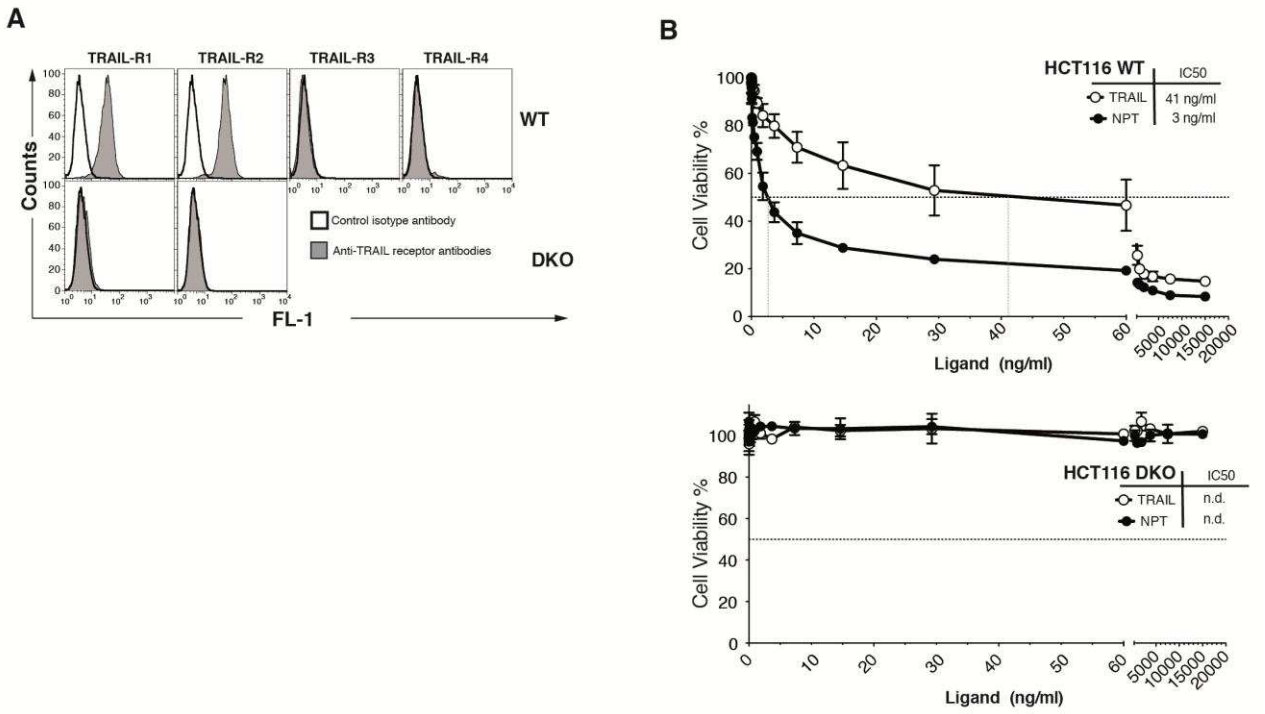


Figure 4.(A) Flow cytometric analysis of TRAIL receptor expression in the Human colon adenocarcinoma cell lines, HCT116 WT and HCT116 DKO. The white histogram represents the isotype control antibody (IgG). The grey histogram corresponds to TRAIL receptor staining. (B) Cell viability in HCT116 WT and HCT116 DKO cells after stimulation with increasing concentrations of TRAIL or NPT. Viability was measured by methylene blue staining. Data are the mean ± SD (n=3).

To further demonstrate that the efficacy of NPT is solely due to TRAIL functionalization on SWCNT but not to the nanotubes themselves, a fixed concentration of TRAIL, NPT or non-functionalized SWCNT (NPP) was used to assess apoptosis in H1703 cells byAnnexin-V staining. As shown by flow cytometry, NPT and TRAIL but not NPP induced apoptosis in H1703 with 90 and 55 % cells undergoing apoptosis 6 hours after stimulation, respectively (Fig. 5A). Incubation of the cells in the presence of the pan-caspase inhibitor ZVAD, 30 min prior stimulation, prevented apoptosis induced TRAIL and NPT (Fig. 5A). Statistical analysis demonstrated that in these cells, apoptosis

inducedby NPT was significantly superior than that induced by TRAIL (Fig. 5B). Similar results were obtained with the parental HCT116cell line (Figure 5C), while the TRAIL-deficient derivative (HCT116-DKO) remainedinsensitive to either TRAIL or NPT (Figure 5C). Irrespective of the cell line, non-functionalized nanoparticles SWCNT-PSE (NPP) were unable to trigger apoptosis.Analysis of TRAIL receptor DISC formation and activation in HCT116 cells stimulated with TRAIL or NPT for 10 to 90 minutes revealed that NPT slightly increased caspase-8 and FADD recruitment to TRAIL receptors (Figure 5D). Importantly, consistent with its superior pro-apoptotic activity, NPT stimulation induced stronger activation of initiator caspases, as evidenced by the increase in caspase-8 p18 or caspase-10 p12 fragments (Figure 5D). Increased activation of initiator caspases upon NPT stimulation, as compared to TRAIL, was also evidenced by the concomitant disappearance of c-$FLIP_L$ proform and the appearance of its p43 cleaved products within the DISC (Figure 5D).These results demonstrate that NPT induces stronger initiator caspase activation within the TRAIL DISC than soluble TRAIL.

Our findings are consistent with earlier reports demonstrating that TRAIL multimerization,[14]in a membrane-like manneris sufficient to increase TRAIL cell killing activity. The grafting of TRAIL on carbon nanotubes efficiently increased TRAIL-induced cell death in various tumor cell types, including HUH and HepG2. It should be noted that these two hepatocarcinoma cell lines express TRAIL-R3 (DcR1) or TRAIL-R4 (DcR2), two TRAIL antagonist receptorslackinga functional death domain, and known to inhibit apoptosis induced by TRAIL to some extent.[8,16, 17]

Inhibition of TRAIL-induced apoptosis in HUH cells by TRAIL-R3 remain, however, unlikely due to the low expression level of this receptor. Yet impairment of TRAIL-induced apoptosis by TRAIL-R4 in HepG2 cells has been documented.[8]Interestingly, NPT was 20 fold more efficient in this cell line, as compared to TRAIL while its efficiency was roughly of 10 fold in the other cell lines. The gain of function in these cells as well as in cells expressing only agonist TRAIL receptors may be

explained by the differential activation of caspase-8 at the DISC level. As demonstrated here after a caspase-8 pull-down, NPT was superior to TRAIL owing to its ability to increase initiator caspase activation (Figure 5D). Increased efficacy of NPT was, however, not associated with increased TRAIL-R1 and TRAIL-R2 pull-down, suggesting that TRAIL functionalization on SWCNT is unlikely to affect TRAIL affinity for its receptors. In contrast, functionalization of TRAIL on SWCNT is more likely to mimic membrane-bound TRAIL and to favor higher order of receptor aggregation, leading to better initiator caspase activation. Whether this gain of function is sufficient to overcome TRAIL antagonist receptor-mediated resistance remains an open question.But the gain of function observed in HepG2 cells, which express spontaneously functional TRAIL-R4, and earlier findings from our group, using sequential treatments associating chemotherapy and TRAIL,[9] suggest that NPT is likely to be able to overcome this resistance thanks to stronger ability to induce caspase-8 activation. Further work will be needed to address this question.

Altogether, we provide here a proof-of-concept that nanovectors based on TRAIL grafted onto SWCNT, can significantly increase TRAIL anti-tumor activity *in vitro*, owing to their ability to increase caspase-8 activation. Likewise, we demonstrate that apoptosis induced NPT is 10 to 20 fold more potent than soluble TRAIL in a variety of tumor cell lines of different origin. Importantly, NPT remained highly selective for TRAIL signaling. Indeed, NPT was unable to trigger apoptosis in the TRAIL receptor-deficient cell line HCT116, generated by the TALEN technology. Moreover, no relevant toxicity due to the nanotubes alone was observed with these cells or other cell line models that we have studied. Given the ability of SWCNT to deliver drugs or to absorb electromagnetic waves, TRAIL functionalized SWCNT may pave the way to novel therapeutic approaches such as NPT-based photothermal therapies or NPT-based TRAIL sensitizing drug delivery.

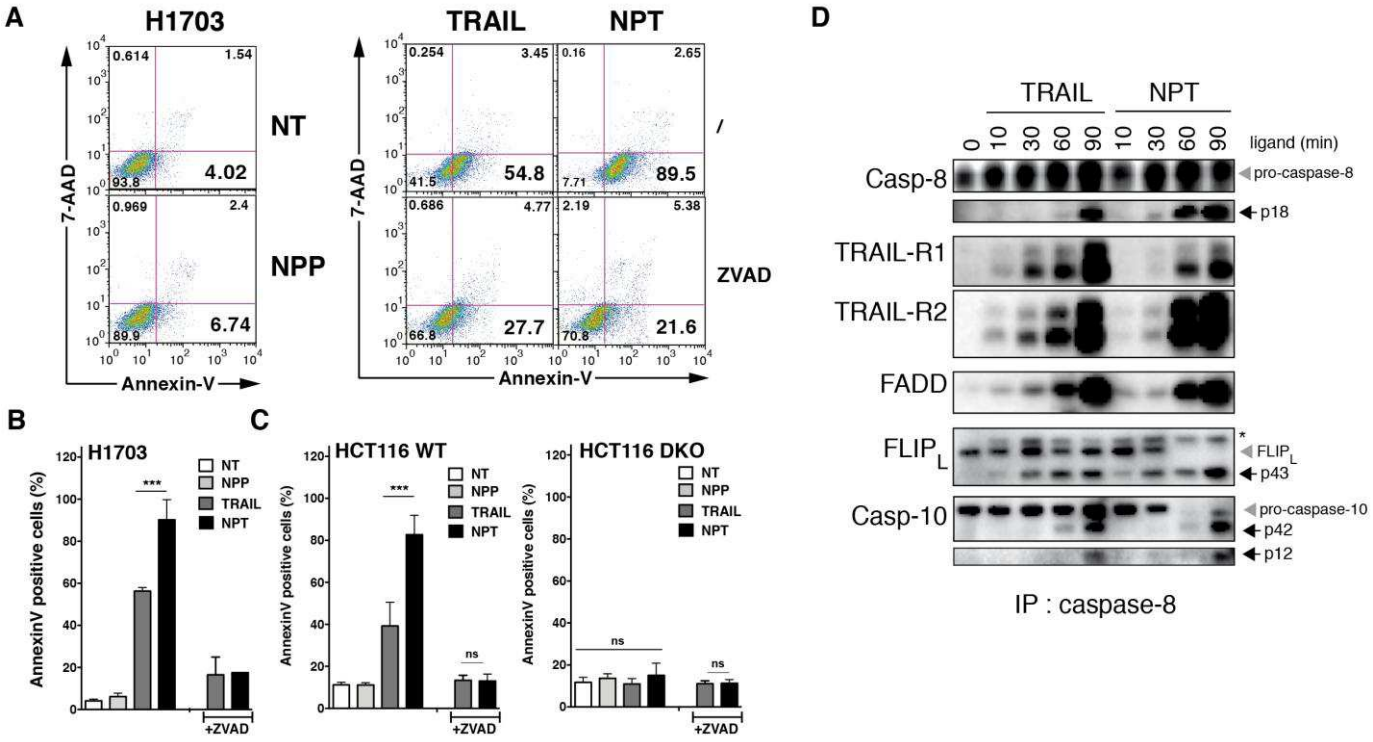


Figure 5. Analysis of cell death by apoptosis (annexin-V) and necrosis (7-AAD) following treatment by the same concentration of NPT and TRAIL (40 ng/mL) in HCT116 cell lines (WT and DKO) and (20 ng/mL) in H1703 cell line. (A) Schematic representation of one result obtained on H1703 cell line with FlowJo software. (B) Percentage of Annexin-V positive cells for H1703 cell lines. (C) Percentage of Annexin-V positive cells for HCT116 cell lines (WT and DKO). Differences between selected groups were compared using non-parametric analysis of variance (ANOVA) with Bonferonni *post hoc* multiple comparison test, ***P < 0.001, compared to TRAIL stimulation alone and NPT, ns (not statistically significant). Data are the mean ± SD (n=3). (D) Immunoprecipitation of caspase-8 and analysis of DISC formation in HCT116-WT cells stimulated with 2 µg/mL His-hTRAIL or NPT for the indicated time periods (see Materials and Methods). After cell lysis in NP40-containing buffer, the DISC was immunoprecipitated (IP) using an anti-caspase-8 antibody and associated proteins were analyzed by Western blotting using antibodies to DR4, DR5, FADD, $FLIP_L$, Caspase-8 and -10 (Casp-8 and Casp-10). Caspase and FLIP cleavage products are indicated by black arrowheads, uncleaved proteins are shown by grey arrowheads. The asterisk shows nonspecific staining bands. Molecular masses are indicated in kDa.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: omicheau@u-bourgogne.fr

*E-mail: guillaume.herlem@univ-fcomte.fr

Author Contributions

O. M.and G. H. contributed equally in this manuscript.

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

Details of the Ligand production, chemical products and antibodies,Single-Walled Carbon nanotubes functionalization with TRAIL, Cell lines & Cell culture, Measurement of cell viability, Annexin-V staining methodology, Hoechst analysis, Spectroscopic measurements, Transmission Electronic Microscopy, Immunoprecipitations, and Western blotting are given in this section.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Table of Contents

Novel nanovector based on SWCNT functionalized with TRAIL via noncovalent 1-pyrenebutanoic acid N-hydrosuccinimid ester and its pro-apoptotic activity.