Published in final edited form as:

Bioconj Chem. 2018 May 16; 29(5): 1659–1668. doi:10.1021/acs.bioconjchem.8b00146.

Immunostimulatory CpG on Carbon Nanotubes Selectively Inhibits Migration of Brain Tumor Cells

Darya Alizadeh¹, Ethan E. White^{2,3}, Teresa C. Sanchez³, Shunan Liu¹, Leying Zhang¹, Behnam Badie^{1,*}, Jacob M. Berlin^{2,3,*}

¹Department of Surgery, Division of Neurosurgery, City of Hope Beckman Research Institute

²Irell & Manella Graduate School of Biological Sciences, City of Hope

³Department of Molecular Medicine, City of Hope Beckman Research Institute, 1500 East Duarte Road, Duarte, CA 91010 USA

Abstract

Even when treated with aggressive current therapies, patients with glioblastoma usually survive less than two years and exhibit a high rate of recurrence. CpG is an oligonucleotide that activates the innate immune system via TLR9 activation. Injection of CpG into glioblastoma tumors showed promise as an immunotherapy in mouse models but proved disappointing in human trials. One aspect of glioma that is not addressed by CpG therapy alone is the highly invasive nature of glioma cells, which is associated with resistance to radiation and chemotherapy. Here, we demonstrate that single-walled carbon nanotubes non-covalently functionalized with CpG (SWNT/CpG), which retain the immunostimulatory property of the CpG, selectively inhibit the migration of glioma cells and not macrophages without affecting cell viability or proliferation. SWNT/CpG also selectively decreased NF- κ B activation in glioma cells, while activating macrophages by induction of the TLR9/NF- κ B pathway, as we have previously reported. The migration inhibition of glioma cells was correlated with selective reduction of intracellular levels of reactive oxygen species (ROS), suggesting that an antioxidant-based mechanism mediates the observed effects. To our knowledge, SWNT/CpG is the first nanomaterial that inhibits the migration of cancer cells while stimulating the immune system.

Corresponding Authors Behnam Badie, Professor, Division of Neurosurgery, 1500 East Duarte Road, Duarte, CA 91010, Phone: 626-471-7100, Fax: 626-471-7344, bbadie@coh.org; Jacob Berlin, Associate Professor, Department of Molecular Medicine, 1500 East Duarte Road, Duarte, CA 91010, Phone: 626-256-4673, Fax: 626-930-5330, jberlin@coh.org.

*Principal Investigators J. Berlin and B. Badie contributed equally

Supporting Information

Additional detailed protocols are available in the supporting information. There are also a number of figures describing our initial finding of potential *in vivo* migration inhibition (Supplementary Figure 1), more detailed characterization of SWNT/LP and SWNT/CpG (Supplementary Figure 2), assessment of migration in K-Luc cells treated with SWNT/CpG and different variants of SWNT/oligos (Supplementary Figure 3), assessment of migration in RAW Macrophages treated with SWNT/CpG and different variants of SWNT/oligos (Supplementary Figure 4), assessment of migration in additional tumor models (GL261, OVCAR8 and HeLa) (Supplementary Figure 5), viability of cells treated with SWNT/CpG constructs (Supplementary Figure 6), wider field of view SEM images of scratch area 22 hrs after treatment (Supplementary Figure 7), characterization of RAW macrophage cellular response to SWNT/CpG treatment (Supplementary Figure 8), SWNT/CpG differentially affects TLR9 and NF- κ B in macrophages and glioma cells (Supplementary Figure 9), additional ROS data (Supplementary Figure 10), SWNT accumulation on cells (Supplementary Figure 11). There are also supplemental live cell videos available showing migration of control and SWNT/CpG-treated K-Luc cells over 18 hrs. These materials are available free of charge *via* the Internet at <http://pubs.acs.org>.

Graphical Abstract

Keywords

single-walled carbon nanotube; CpG; glioma; macrophages; migration; Reactive Oxygen Species

Introduction

Gliomas account for 80% of all malignant primary tumors in the brain and central nervous system.¹ Glioblastoma is the most common form of glioma and despite surgery, chemotherapy, and radiation, the median survival for these patients is less than 2 years and the 5-year survival rate is 10%.¹⁻³ Glioblastoma patients also exhibit high rates of tumor recurrence partly due to the invasive nature of the disease.² Although recent advances are encouraging, there is still a need to develop new, more effective treatments.^{4, 5}

As demonstrated by the clinical success of checkpoint inhibitors, an endogenously generated anti-tumor immune response has great potential for cancer therapy.^{6, 7} One mechanism for achieving this endogenous anti-tumor response is stimulation of immune cells using Toll-Like Receptor (TLR) agonists and other adjuvants, either as a monotherapy or in combination.⁸⁻¹⁰ However, the use of TLR agonists for cancer immunotherapy is a “double-edged sword”.¹¹ Stimulation of TLRs has been associated with increased proliferation, metastasis, and drug resistance in some cancers.^{11, 12} In particular, CpG, which is a DNA oligonucleotide containing unmethylated CG motifs, is well-known for eliciting a Th1 immune response by inducing the production of inflammatory cytokines and type 1 interferon in immune cells, mediated in part by activating TLR9 which leads to activation of the NF- κ B transcription factor.¹³ In glioma, though, CpG-mediated TLR9 stimulation is reported to cause increased invasiveness and promote the maintenance of cancer stem cells.^{14, 15} For cancer immunotherapy, it would be beneficial if the activity of CpG were modulated so that it retained its immune stimulation ability but no longer elicited pro-tumor effects.

Previously, we demonstrated that CpG noncovalently conjugated to single-walled carbon nanotubes (SWNT/CpG) has greater immunostimulatory potential than free CpG, strongly activating both immortalized and primary macrophages, and exhibits efficacy in multiple cancer immunotherapy models.¹⁶⁻¹⁸ In one mouse model of glioma, using GL261 cells, a

single low-dose intracranial (i.c.) injection of SWNT/CpG (but not free CpG nor the SWNT control) eradicated GL261 gliomas in 50–60% of mice.¹⁶ In a recent report, we found that the efficacy of temozolimide, the current standard of care chemotherapy for glioblastoma, was enhanced when combined with SWNT/CpG immunotherapy for treating an orthotopic mouse model of glioma. Tumors were established in this model using K-Luc cells, a murine glioma cell line derived from spontaneous gliomas in *Trp53/Nf1* double-mutant mice that forms a highly-invasive tumor.¹⁷ Following that report, we continued to evaluate the tumor histology of control and treated mice and observed that SWNT/CpG appeared to locally alter the invasive properties of the tumor (Supplementary Figure 1). This suggested to us that this formulation might be achieving the desired modulation of CpG activity, promoting an anti-tumor and pro-immune response.

Inhibiting migration and invasion of cancer cells has been the focus of many investigations.¹⁹⁻³⁰ In general, the majority of these inhibitors are small molecules with relatively few reports of nanoparticle-based inhibitors and no previous reports implicating either SWNTs or CpG as migration inhibitors.³¹⁻³⁸ In glioma, infiltration of cancer cells throughout the brain tissue is thought to be a major reason for the high rates of recurrence.³⁹⁻⁴¹ Furthermore, the migratory phenotype has been associated with resistance to treatment and decreased apoptosis in glioma, suggesting that migration inhibitors may also have the potential to interfere with pro-tumor pathways.^{34, 39, 41-52} There has been very limited research into combining cancer migration inhibition with immunotherapy and, because most migration inhibitors are only tested with cancer cells, it is generally unclear what effects they may have on immune cells.

In this report, in order to evaluate if SWNT/CpG indeed had a direct effect on glioma cell migration, we use an *in vitro* model of cell migration. We found that the SWNT/CpG construct inhibited the migration of glioma cells while maintaining the immunostimulatory properties seen in our previous publications. In glioma cells, migration inhibition was accompanied by decreased NF- κ B activation as well as an overall reduction in intracellular ROS. In macrophages, SWNT/CpG increased activation of the TLR9/NF- κ B pathway without impairing migration. The cell type-specific activity of SWNT/CpG has considerable implications for the design of combination brain tumor therapies that simultaneously tackle the migration of glioma cells while inducing immune activation in the tumor microenvironment.

Results and Discussion

Our previous work has focused on the ability of SWNT/CpG to activate macrophages and induce an anti-tumor immune response in mouse models of glioma.¹⁶⁻¹⁸ These experiments have extensively characterized the pro-inflammatory effects of SWNT/CpG treatment on macrophages, both *ex vivo* with immortalized macrophage cell lines and human PBMCs as well as *in vivo* in tumor bearing mice. In these reports, it was shown that SWNT/CpG was not directly toxic to glioma cells (further validating that anti-tumor efficacy arises from an immune activation). More extensive characterization of the effects of SWNT/CpG on cancer cells was not conducted as it was presumed that the primary mode of action for SWNT/CpG was immune activation. However, in our most recent efficacy study, histological images of

tumors from SWNT/CpG-treated and untreated tumor-bearing mice suggested that treatment with the nanoparticle-based cancer immunotherapy decreased the migratory ability of the highly invasive murine K-Luc gliomas (Supplementary Figure 1).¹⁷ Motivated by this unexpected observation, we sought to further investigate and validate this observation in more detailed *in vitro* studies.

We previously tested several strategies for conjugating CpG to the SWNTs,^{16, 17} and active formulations consisted of SWNTs coated with a complex mixture predominately containing modified CpG and a lipid-PEG construct. Starting from this more complex formulation, here we generated a series of simplified variants to determine the minimal formulation necessary for activity (migration inhibition studies for this library of variants is partially shown in Supplementary Figures 3 and 4). We found that simply dispersing SWNTs in CpG yields a material (SWNT/CpG, Figure 1A) with similar immunostimulatory activity as was seen in our previous studies (increasing NF- κ B activity ~2-fold in treated RAW-Blue macrophages, an NF- κ B reporter line derived from RAW 264.7, Fig 1B).^{17, 18} The CpG DNA likely binds to the SWNT surface through noncovalent interactions as has been previously reported for other single-stranded DNA used to prepare aqueous-stable dispersions of SWNTs.⁵³⁻⁵⁵ This simplified SWNT/CpG construct is easier to prepare than our previous formulations which is important for both synthesizing larger amounts and ensuring the material is free of immunostimulatory contaminants (such as endotoxin) that could confound the results. To ensure this, the SWNTs were autoclaved after massing and stored under sterile conditions prior to use, and the CpG starting material was sterile-filtered and endotoxin-tested. Since SWNTs alone cannot be dispersed in water, a control material was prepared by coating SWNTs with a lipid-PEG conjugate (SWNT/LP, Figure 1A) as this conjugate also binds to the SWNT surface noncovalently and yields bundled SWNTs of a similar size that are dispersible in aqueous solutions (Supplementary Figure 2).⁵⁶ It is difficult to accurately measure the size of SWNT constructs, particularly in solution, so we compared the sizes of SWNT/LP and SWNT/CpG by transmission electron microscopy (TEM) analysis, which involved imaging 3-4 batches of each material and randomly selecting one field of view for each batch to manually measure length and width of bundles. Both materials are very heterogeneous across all batches, but by comparing histograms of the lengths and widths measured it appears that the two materials are similar in size and SWNT/LP is an appropriate control for the effect of SWNT in solution.

We proceeded to test the impact of treatment with CpG, SWNT/LP or SWNT/CpG on K-Luc cells, the highly invasive murine glioma line. For the glioma cells, SWNT/CpG inhibited the migration of K-Luc cells while both CpG and SWNT/LP had minimal effect (Figure 1C and Supplementary Video 1-2). Interestingly, migration inhibition was only observed when SWNT was sonicated with CpG. When K-Luc cells were treated with a mixture of both CpG and SWNT/LP, this co-treatment was not able to recapitulate the migration inhibition observed with SWNT/CpG (Supplementary Figure 3). This finding suggests that CpG and SWNT act as a noncovalently-bound complex and are unable to inhibit glioma migration when acting independently.

We also evaluated the effect of treatment on mouse macrophage cells. Our previous work has consistently demonstrated the ability of CpG conjugated to SWNTs to activate

macrophages, ranging from immortalized mouse macrophages to freshly isolated human peripheral blood mononuclear cells (PBMCs).^{16, 17, 57-66} Here, we chose to test immortalized mouse macrophages since our initial *in vivo* observation of potential glioma cell migration inhibition was in mice and all of our previous efficacy studies were in mice. In RAW-Blue cells, SWNT/CpG treated cells showed no evidence of cell migration inhibition, indicating that inhibitory effects of SWNT/CpG are cell type-specific and not a general cell phenomenon (Figure 1D). CpG and SWNT/CpG appeared to have a similar effect promoting migration of macrophages.

We next evaluated if the observed cancer cell migration inhibition was restricted to K-Luc cells. We found that SWNT/CpG also inhibits the migration of several other cancer cell lines: the less invasive GL261 mouse glioma cell line, the human ovarian cancer cell line OVCAR8 and the human cervical cancer cell line HeLa (Figure 2A and Supplementary Figure 5). For the control untreated condition, each of these cell lines required different timing for the cells to fill in the scratch, so the treated cells were imaged at these different time points (Figure 2A, see Supplementary Methods for more details on optimizing the assay for each cell line). We also found that the effect was not restricted to 2D cell culture as SWNT/CpG-treated K-Luc cells exhibited limited migratory capacity in a 3D culture assay (Figure 2B).

To investigate the mechanism by which SWNT/CpG inhibits glioma cell migration, we first looked for obvious changes in cell viability, proliferation and morphology. SWNT/CpG treatment showed no effect on the viability (assessed by LIVE/DEAD staining, Figure 3A and DMSO-treated positive control Supplementary Figure 6) or morphology (assessed by SEM, Figure 3A and Supplementary Figure 7) of K-Luc glioma cells. SWNT/CpG also had no effect on the proliferation of the K-Luc glioma cells (Figure 3B). Furthermore, when fluorescently labeled CpG^{Cy5.5} or SWNT/CpG made with CpG^{Cy5.5} were incubated with K-Luc for 22 hours and then imaged by confocal microscopy, no apparent differences in cell morphology (actin staining) or sub-cellular localization of CpG were observed (Figure 3D). Similar findings were observed in treated RAW macrophage cells (Supplementary Figure 8).

Having observed no impact on viability, morphology or proliferation, we continued our mechanistic investigation by evaluating the impact of SWNT/CpG on NF- κ B and TLR9 expression. CpG is a known TLR9 agonist and NF- κ B is a downstream transcription factor of TLR9. Here, we sought to measure the effect of SWNT/CpG on NF- κ B and TLR9 in cancer cells as constitutive activation of NF- κ B is found in many human tumors, including glioma⁵⁷, and contributes to tumor cell survival, proliferation, migration, and therapeutic resistance.⁵⁸⁻⁶¹ TLR9 expression is correlated with a poor clinical prognosis for glioma patients and TLR9 stimulation has been shown to increase the invasiveness of glioma cells *in vitro*.^{14, 62} In contrast to some studies suggesting that CpG therapy may induce TLR9 expression in tumor cells¹⁵, and in contrast to the effect on macrophages, neither SWNT/CpG nor CpG induced TLR9 expression in K-Luc glioma cells (Figure 4A, Associated fluorescent microscopy images are in Supplementary Figure 9). Indeed, the observed migration inhibition was not dependent on TLR9 activation by CpG as treatment of K-Luc cells with SWNT/RSS-GpC also resulted in migration inhibition comparable to that achieved with SWNT/CpG and inverting the CpG di-nucleotide motifs to GpC is known to

abolish TLR9 activation (Supplementary Figure 3).⁶³ Moreover, SWNT/CpG actually decreased NF- κ B expression in K-Luc glioma cells (Figure 4B). Untreated K-Luc cells exhibited high nuclear localization of NF- κ B p65 subunit indicative of a constitutive NF- κ B activation, but this staining was markedly reduced by SWNT/CpG treatment (Supplementary Figure 9). Collectively, SWNT/CpG treatment elicited contrasting cell type-specific responses, increasing TLR9 and NF- κ B expression in macrophages while having little impact on TLR9 and decreasing NF- κ B expression in glioma cells.

We next sought to understand what material property of SWNT/CpG led to this cell type-specific response. Increased reactive oxygen species (ROS) levels have been correlated with increased activation of NF- κ B in many different cell types.^{64, 65} Increased ROS levels have been reported as an important factor promoting the invasive properties of several cancer types including gliomas.⁶⁶⁻⁶⁹ Moreover, SWNTs are known to possess antioxidant properties⁷⁰, so we hypothesized that the ability of SWNT/CpG to inhibit glioma cell migration and down regulate NF- κ B in these cells could be due to the SWNT/CpG's ability to decrease ROS. We measured ROS levels by treating cells with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CMH₂DCFDA), which is only fluorescent following oxidation. This pro-dye broadly measures the level of ROS in a cell, with limited or no specificity for one type of ROS over another. We found that K-Luc cells treated with SWNT/CpG exhibited lower cellular ROS levels as compared to untreated cells or to cells exposed to CpG or SWNT/LP (Figure 5A, Supplementary Figure 10). Interestingly, there was no apparent decrease in ROS levels for SWNT/CpG-treated macrophages (Supplementary Figure 10). To determine if reduced intracellular ROS was sufficient to cause migration inhibition, K-Luc cells were treated with N-Acetyl Cysteine (NAC), a known ROS scavenger (Figure 5B). As hypothesized, the migration of K-Luc cells was reduced by NAC treatment (Figure 5C). Of note, much higher doses of NAC were required to inhibit glioma cell migration than for the SWNT/CpG. NAC treatment also did not exhibit cytotoxic effects and did not impair cell proliferation (Supplementary Figure 10). While ROS reduction was observed for both SWNT/CpG and NAC at concentrations where they inhibited K-Luc cell migration, it is unclear if ROS reduction causes migration inhibition or is merely correlated with it. More detailed molecular biology studies will be required to determine this relationship.

Overall, these findings demonstrate that SWNT/CpG maintains the immunostimulatory properties of CpG while eliminating a primary mode of pro-tumor activity. To the best of our knowledge, SWNT/CpG's ability to activate immune cells and inhibit the migration of cancer cells is unique. This dual mode of action has the potential to benefit glioma therapy by simultaneously addressing the immunosuppressive and highly invasive nature of glioma, both of which contribute to the difficulty in treating it. Our working hypothesis is that immune cell activation is primarily driven by TLR9 activation involving CpG while cancer cell migration inhibition is primarily driven by the antioxidant capacity of the SWNTs. We hypothesize that migration inhibition is only observed for SWNTs functionalized with oligonucleotides and is not observed for LP functionalization because the oligonucleotide coating alters the localization of the SWNTs, resulting in either greater interaction with the cells or different localization within the cells – or both (Supplementary Figure S11). While these results are promising, SWNT materials have historically proven extremely challenging

to translate into the clinic. Further studies will involve both molecular biology studies to elucidate the mechanism of action for migration inhibition by SWNT/CpG and studies of various alternative surface coatings for the SWNT to better understand what material properties are required. The eventual goal is to use this mechanistic and material knowledge to develop novel clinically translatable materials that can serve as dual immune system stimulants and glioma cell inhibitors

Materials and Methods

Reagents

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (Lipid-PEG-OME) was purchased as an ammonium salt in powder form from Avanti Polar Lipids, Inc. Ultra pure SWNTs (<1% metal impurities) were purchased from NanoIntegris in powder form.

All oligonucleotides are fully phosphorothioated and were both synthesized and HPLC-purified by IDT (the initial batch for pilot testing, used in Supplementary Figure 3) or the DNA/RNA Synthesis core facility at the Beckman Research Institute at City of Hope (all other samples). *CpG*: (5'-

T*A*A*A*C*G*T*T*A*T*A*A*C*G*T*T*A*T*G*A*C*G*T*C*A*T-3')⁷¹⁻⁷⁴ *GpC*: (5'-T*A*A*A*G*C*T*T*A*T*A*A*G*C*T*T*A*T*G*A*G*C*T*C*A*T-3'). The "RSS-" variant of the oligonucleotides (RSS-CpG and RSS-GpC) contained a C6 thiol modification on the 5' end. The fluorescently-labeled CpG (CpG^{Cy5.5}) was modified on the 3' end with Cyanine5.5.

Sample Preparation

CpG was dissolved in water under sterile conditions to make a stock solution. This stock solution was passed through a 0.22 µm filter and tested for endotoxin. To make SWNT/CpG, sterile CpG stock solution was added to SWNTs under sterile conditions. SWNTs and CpG were combined in a 1:1 mass ratio, regardless of batch size, and sonicated to make a stable dispersion (cup horn sonicator, QSONICA Sonicator Q700). After sonication, the sample was diluted to 1 mg/mL SWNT and 1 mg/mL CpG using sterile MilliQ water. SWNT/LP was made by dispersing the SWNTs in a sterile stock solution of Lipid-PEG-OME using the same procedure as SWNT/CpG. For a more detailed procedure, see the Supplementary Methods.

Endotoxin Test

Endotoxin testing was performed by the Department of Immunology at City of Hope using the Endosafe Portable Test System (Charles River Labs) and a 0.005 EU/mL sensitivity Endosafe - Licensed PTS Endotoxin Cartridge (catalog no. PTS20005F, Charles River Labs) following the manufacturer's instructions. Only samples containing <0.4 EU/mL were used for experiments. For a more detailed procedure, see Supplementary Methods.

Cell line and cultures:

The K-Luc murine glioma cell line (C57BL/6 origin, Luciferase-expressing KR158B cells [K-Luc]) was a generous gift from Dr. John Sampson.⁷⁵ The GL261 murine glioma, OVCAR8 human ovarian cancer, and HeLa human cervical cancer cell lines were generous gifts from Dr. Karen Aboody. RAW-Blue™ cells were purchased from InvivoGen. RAW-Blue™ cells are RAW 264.7 murine macrophages with a stably integrated SEAP reporter for NF- κ B and AP-1. K-Luc, RAW-Blue™, and GL261 cells were cultured and maintained in DMEM (gibco, +4.5 g/L D-glucose, +L-glutamine, +110 mg/L sodium pyruvate) supplemented with 10% FBS, (BioWhittaker), 100 U/mL penicillin-G, 100 μ g/mL streptomycin and 0.01 M HEPES buffer (Life Technologies). OVCAR8 cells were cultured in RPMI 1640 (gibco, - L-glutamine) supplemented with 10% FBS, 1mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. HeLa cells were cultured in DMEM (gibco, +4.5 g/L D-glucose, +110 mg/L sodium pyruvate, - L-glutamine) supplemented with 10% FBS, 1mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml. All cells were grown in a humidified 5% CO₂ atmosphere at 37°C.

Light Microscopy:

Widefield images and video of the 2D migration assay and the 3D dot migration assay were obtained on an Axio Observer Z1 Inverted Microscope (Zeiss) equipped with a Pecon / Zeiss Incubation System using Zen2 Blue software. During imaging, the 5X/0.16NA Plan-Neofluar Phase objective and the Hamamatsu EMCCD C9100-13 Monochrome Camera were used. Incubation conditions for the live cell videos were 5% CO₂ and 37°C with a heated stage insert. For fluorescent images of the viability assay, the filter sets were #38 Ex; BP470/40, Beam Splitter; FT495, Emission; 525/50 (eGFP) and #43 Ex; BP545/25, Beam Splitter; FT570, Emission; 605/70 (DS Red). To detect intracellular protein or labeled CpG and SWNT/CpG, confocal microscopy was performed using a LSM 700 Meta (Zeiss).

2D Migration assay and 3D migration assay:

For 2D migration assay, cells were cultured within the insert chambers of the 35 mm u-dish (ibidi). Inserts were removed, the monolayer was rinsed with media to remove floating cells, and images were taken both before treatment and at the indicated time points after treatment. Cells were seeded at $2.0\text{--}2.5 \times 10^4$ (K-Luc, GL261 and RAW-Blue™), 1.5×10^4 (HeLa), or 8.0×10^3 (OVCAR8) cells per insert chamber. For a more detailed procedure, see Supplementary Methods.

For 3D migration assay, total of 1×10^6 tumor cells were labeled with PKH26 (sigma) and treated with nothing (control), SWNT/LP, CpG, or SWNT/CpG in suspension with culture medium containing 3% matrigel (Corning Inc.). A total of 5 μ l of the mixture was placed at center of the well in a form of a drop. After 3 hours, when cells formed a 3D colony in the matrigel, each well was covered with a layer of culture medium containing 1% matrigel. Images were taken on day 1 and day 3 post-3D formation.

Viability assay:

To measure viable cells, cells were evaluated based on staining with green-fluorescent calcein-AM (Thermofisher) to indicate intracellular esterase activity (live cells) and red-

fluorescent ethidium homodimer-2 (EthD-2) (Setareh Biotech, 1mM solution in DMSO) to indicate loss of plasma membrane integrity (dead cells). As a positive control for staining, cells were treated with either 10% DMSO overnight (in incubator) or 70% ice-cold ethanol for 30 minutes (room temperature). Cells were stained with warmed complete media containing 1µM calcein-AM and 2µM EthD-2 for 30-60 minutes at 37°C.

In vitro NF-κB assay:

RAW-Blue™ mouse macrophage reporter cells (Invivogen) were cultured to measure TLR-mediated immune stimulation following the manufacturer's protocol. Briefly, cells (5000 cells in 100 µL in a 96-well dish) were culture overnight and then treated with PBS (1 µl), CpG (1 µl of 1 µg/ul stock) SWNT/LP (1 µl of 1 µg/ul stock) or SWNT/CpG (1 µl of 1 µg/ul stock) for 16 hours. The level of secreted embryonic alkaline phosphatase (SEAP) was quantified using QuantiBlue™ substrate (InvivoGen) and measured after 2 hours at 620 nm absorption using DTX 880 Multimode Detector (Beckman Coulter). QUANTI-Blue substrate was prepared according to manufacturer's protocol (InvivoGen).

Proliferation assays:

K-Luc cells were labeled with 5mM CFSE using the CellTrace™ CFSE cell proliferation kit according to the manufacturer's instructions (Invitrogen). At indicated time points, cells were collected and proliferation was determined by measuring the dilution of CFSE by flow cytometry. The proliferation index (pi) was determined using the Modfit software (Verity Software House) and percent proliferation was calculated as follows: proliferation (%) = $\frac{(T^+ - T) - (S - T)}{(T^+ - T)} \times -100$, with T⁺: pi of the control tumor cells after proliferation; T: pi of control tumor cells before proliferation; S: pi of treated tumor cells).

Immunofluorescent staining:

K-Luc cells or RAW-Blue™ macrophages were cultured and treated at various time points. Cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature (RT) following 10 minutes in 100% cold methanol. After blocking for 1 hour at RT in 1% BSA, cells were incubated with primary antibody NF-κB p65 (1:100 in TBST Santa Cruz Biotech) for 1 hour. Cells were washed with Tris-Buffered Saline and 0.1% Tween 20 (TBS-T) and incubated with a secondary chicken anti-rabbit antibody conjugated with Alexa Fluor 558 (Invitrogen) for 1 hour at RT (1:500 in TBST, Invitrogen) following three washes with TBS-T. For TLR9 staining, cells were labeled with TLR9 conjugated FITC antibody (1:100 in TBST, Affymetrix eBioscience). All slides were mounted and counterstained with DAPI using Vectashield (Vector Laboratories). Slides were imaged using confocal microscope (Zeiss LMS700).

For uptake analyses, cultured K-Luc or RAW-Blue™ macrophages were incubated with labeled CpG^{Cy5.5} or SWNT/CpG^{Cy5.5} at indicated time points. Cells were fixed (3.7% paraformaldehyde in PBS) for 10 minutes. Cells were washed with PBS following 5-minute incubation with permeabilization buffer (0.5 % Triton X-100 in PBS). Cells were washed twice with PBS then incubated with 100 nM Acti-stain 488 phalloidin (Cytoskeleton, Inc) at RT for 30 minutes. Cells were washed three times with PBS following DAPI stained and mounted using Vectashield (Vector Laboratories).

Reactive oxygen species (ROS) detection:

K-Luc tumor cells or RAW-Blue™ macrophages from untreated or treated with CpG, SWNT/LP, CpG/SWNT were incubated for 30 min with the oxidation-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CMH₂DCFDA, 5μM, Molecular Probes/Invitrogen). Samples were then collected and washed with PBS. The level of ROS was detected by measuring fluorescence intensity with flow cytometry at an excitation wavelength of 488 nm and emission at a wavelength of 530 nm. Data was collected and analyzed using Fortessa (BD biosciences) and Flowjo (Flowjo, LLC).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was performed on an FEI Quanta 200 scanning electron microscope. Cells were cultured in a standard ibidi μ-Dish (Ibidi, #81176) used for the 2D migration assay and fixed with 2% glutaraldehyde in 0.1M Cacodylate buffer (Na(CH₃)₂AsO₂ · 3H₂O), pH7.2, at 4°C, overnight. The polymer coverslip was peeled-off of the dish, washed three times with 0.1M Cacodylate buffer, pH7.2, post-fixed with 1% OsO₄ in 0.1M Cacodylate buffer for 30 min and washed three times with 0.1M Cacodylate buffer. The samples were then dehydrated through 60%, 70%, 80%, 95% ethanol, 100% absolute ethanol (twice). The samples were dried in a critical-point dryer and then coated with gold and palladium (Au: Pd 60/40 ratio) in a Cressington 308R coating system.

Statistical Analysis:

Statistical analyses were performed with the prism software using the unpaired, two-tailed Student t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We gratefully acknowledge Marcia Miller, Zhuo Li, and Ricardo Zerda for electron microscopy performed in the EM core facility; Brian Armstrong, Loren Quintanar, and Tina Patel for their assistance with fluorescent imaging performed in the Light Microscopy and Digital Imaging Core; David Colcher and Jing Guo in the Department of Immunology for performing endotoxin testing; and Jacob White for assistance with R Studio software. Research reported in this publication included work performed in the Analytical Cytometry Core, Integrated Genomics Core and Drug Discovery & Structural Biology Core (DNA/RNA Synthesis Core) supported by the National Cancer Institute of the National Institutes of Health under award number P30CA033572. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors would like to thank R01CA155769, R21CA189223, R21 NS081594, The Kenneth T. and Eileen L. Norris Foundation and STOP Cancer for research funding. TC Sanchez was supported by an internship funded by grant R25 OD010513 from the Science Education Partnership Award program at NIH.

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Figure 1. Preparation and evaluation of SWNT/CpG.

A) SWNT/CpG and SWNT/LP are prepared by sonicating SWNT with the appropriate coating agent (CpG or Lipid-PEG). B) SWNT/CpG increases NF- κ B activation in macrophages relative to CpG alone ($p < 0.0001$ for both CpG vs SWNT/CpG and SWNT/LP vs SWNT/CpG). C) SWNT/CpG inhibits the migration of glioma cells in a scratch assay and D) does not inhibit the migration of macrophages. CpG and SWNT/CpG appear to have a similar effect promoting migration. Scale bar applies to C) and D).

Figure 2. Generality of SWNT/CpG migration inhibition.

A) SWNT/CpG inhibits the migration of a less invasive mouse glioma cell line (GL261), a human ovarian cancer cell line (OVCAR8) and a human cervical cancer cell line (HeLa). Timing to best observe the effect varies by cell line. B) SWNT/CpG inhibits K-Luc migration in a 3D culture.

Figure 3. SWNT/CpG does not perturb cell survival, proliferation or morphology.

K-Luc cells were treated exactly as they were for migration studies and then assayed for viability, proliferation and cell morphology at 22 hrs, which is the timepoint shown for the migration studies. A) Viability and morphology of K-Luc cells were not affected by the treatments as evident by live/dead staining and SEM, respectively. B) Proliferation of K-Luc cells was not impaired by the treatments. D) Cell morphology is unchanged by SWNT/CpG treatment as compared to CpG treatment and the subcellular localization of CpG is similar in both cases.

Figure 4. Additional measurements of TLR9 and NF- κ B activation.

(A) K-Luc and RAW macrophages were treated, stained with TLR9-specific antibody and then the signal was quantified by flow cytometry. (B) K-Luc cells were treated with CpG or SWNT/CpG and NF- κ B(p65) expression was measured at various time points by western blot.

Figure 5. SWNT/CpG functions as an antioxidant in glioma cells and ROS ablation results in migration inhibition.

A) SWNT/CpG reduces ROS levels in glioma cells. (SWNT/CpG vs Control $p < 0.0001$). B) Similar reduction in ROS levels and C) migration inhibition is achieved by treatment with high concentrations of NAC, a small molecule antioxidant.