Research Article


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Cell type, morphology, and functioning are key variables in the construction of efficient “drug-vehicle” hybrids in magnetic drug delivery. Iron-encapsulated multiwall carbon nanotubes (Fe@MWCNTs) appear as promising candidates for theranostics due to in situ chemical catalytic vapor deposition (c-CVD) synthesis, straightforward organic functionalization, and nanoneedle (1D) behavior. Here, model hybrids were synthesized by exploring C-sp2 chemistry ((1+2)-cycloaddition of nitrenes and amidation) of the outer MWCNT walls combined with anticancer agents, that is, 5-fluorouracil (5FU), purpurin (Purp), and 1,8-naphthalimide DNA intercalators (NIDIs), via linkers. Analyses of the Fe@MWCNT vehicles by SEM, TEM, and Raman spectroscopy revealed their morphology while Mössbauer spectroscopy confirmed the presence of encapsulated ferromagnetic iron-based nanodomains. Cytotoxicity of the hybrids was studied using a 24 h MTS assay combined with the apoptosis and life cycle assays against human melanoma (Me45), colon carcinoma (HCT116+), and colon adenocarcinoma (Caco-2). The cells had different sensitivity to the vehicles themselves as well as to the hybrids. MWCNT-based covalent hybrids of 5FU and Purp emerged as the most promising systems against Me45 and HCT116+ cell lines with the highest in vitro cytotoxicity and proapoptotic activity. Furthermore, nanotubes bearing 4-nitro- and 4-(N-morpholinyl)-1,8-naphthalimide DNA intercalators appear as a promising candidate for the treatment of Caco-2.

1. Introduction

With the constantly growing number of new cases worldwide, cancer diseases continue to require more selective and more efficient therapies [1, 2]. Among a variety of types, colorectal cancer is the third most frequent cancer accounting for 10% of all cases worldwide while melanomas develop with the highest rate of new incidents [3]. A recent rise of nanotechnology has poured new hopes to conquering cancer and the research with the “nano” descriptor yielded numerous successful implementations of nanomedicine to medical practice [4]. Nanomedicine exploits therapeutic and/or diagnostic (or combined “theranostic”) agents as the key field of commercialization of anticancer products [5]. And here, locoregional therapy using magnetic drug delivery systems (DDSs) emerges as one of the most promising approaches to cure
cancer [6]. DDSs are composed of therapeutic agents attached to the surface of magnetic nanoparticles or encapsulated within a nanocomposite mixture of a polymer and magnetic nanoparticle. Ideal properties for DDSs cover full biocompatibility, high magnetization, and minimal magnetic remanence at the removal of magnetic field (reducing aggregation and enabling fast excretion). This approach with numerous in vivo studies exploits, for example, various polymer-drug conjugates based, for example, on polyethylene glycol (PEG), N-(2-hydroxypropyl)methacrylamide (HPMA), polymeric micelles, and nanoparticle carriers which can be designed with respect to absorption, distribution, metabolism, and excretion (ADME scheme) of numerous anticancer drugs. And so, in one of the more distinguished examples, a doxorubicin-Fe$_3$O$_4$ complex was prepared by coordination chemistry in which an “interconnecting” Fe$^{2+}$ cation served as a bridge between phenolic -OH groups of the drug molecule and superparamagnetic surface of Fe$_3$O$_4$, and the hybrid was covered by PEGylated surface-OH group [7]. In another variant, silica-coated Fe$_3$O$_4$ nanospheres were coated with redox-responsive cyclodextrin molecules and, after their uptake by cells, high intracellular glutathione concentration caused the pores to become unblocked, liberating the drug [8]. In another example of magnetically targeted therapy, Liu and coworkers have developed Fe$_3$O$_4$ nanoparticles encapsulated within a poly(aniline-co-N-(I-one-butryc acid)) outer shell and epirubicin was immobilized on the polymer coating. The hybrid was then targeted to the brain using focused ultrasound and magnetic targeting as a synergistic delivery system [9].

Colon carcinomas [10] and melanomas [11] are composed of cells of various morphologies and frequently undergo dangerous metastasis [12]. While response to chemotherapeutics can strongly vary, rapid growth of the cells typically corresponds to drug sensitivity [13]. Hence, on the one hand, when designing targeted therapy systems, both chemical structure of a drug and parameters of its loadable vehicle (frequently forming a multifunctional hybrid) must be addressed. On the other hand, morphology, functioning, and proliferation rate of the cells are a highly changeable drug target. Such a set of variables requires enhanced uptake of “drug-vehicle” hybrids as quickly maneuverable “Trojan horses,” by definition fully loaded with cleavable chemotherapeutic agents and safely excretable after unloading.

In the midst of many magnetic nanoparticles applicable in medicine, iron-encapsulated multiwall carbon nanotubes (Fe@MWCNTs) emerge as powerful drug vehicles mainly due to convenient functionalization and needle-like permeation of cells [14]. Nevertheless, continuously disputable concerns in pulmonary toxicity of long, nonfunctionalized, and nonindividualized MWCNTs [15] led to delays in clinical trials of systems based on nanotubes. On the other hand, today, there is no doubt that short, functionalized with polar moieties, and individualized MWCNTs offer a hope to be tested clinically [16]. But earlier in vitro [17] and in vivo [18] reports confirmed that, after functionalization, even >40 μm long Fe@MWCNTs, providing individualized (i.e., not aggregated) nanotubes, could be considered as enzymatically degradable [19, 20] and hence excretable drug vehicles [21].

Although significant progress in the field of “nanotube against cancer” has been already made and extensive research continues, some of the key challenges remain [22]. And, apart from mechanisms of in vivo cytotoxicity, biodistribution, effective targeting issues, and routes of the MWCNT excretion, the most critical of them is to (1) design new routes of mild and efficient “drug-to-nanotube” tethering, (2) identify selective anticancer “drug-nanotube” hybrids towards specific cells, and (3) reveal their potential in short period therapies [23].

Pure MWCNTs are diamagnetic [24] and their magnetic steerability must be achieved by combining with ferro- or superparamagnetic components [25–27], for example, by decoration with Fe$_3$O$_4$ [28] or Fe encapsulation (at the stage of c-CVD synthesis or via capillary forces as a posttreatment) [29]. Indeed, as c-CVD is a convenient and tunable one-step synthesis of Fe@MWCNTs, we decided to focus on effective loading of Fe@MWCNTs with chemotherapeutics representing various mechanisms of cytotoxicity and to anchor the drugs using different chemical routes. In “nonmagnetic” but otherwise targeted delivery, MWCNTs have served as vehicles for strong cytostatic drugs, for example, cisplatin [30, 31]. E.g. Bhirde et al. have shown that MWCNTs-EGF-cisplatin quickly reached the squamous carcinoma cells and significantly reduced their proliferation [32]. However, as cisplatin selectively attacks squamous cells, much more attention was devoted to anthracycline drugs, particularly doxorubicin (Dox) which exhibits a broader spectrum of activity [33, 34]. Purpurin (Purp) displays a similar activity to Dox [35]. It is an active compound in photodynamic therapy [36] and demonstrates an ability to inhibit topoisomerase II as well as intercalate DNA [37]. In addition, its latent hydroquinone moiety may be metabolized generating a large number of free radicals damaging both DNA and cell membrane. Hence, Purp is currently considered as the important alternative of Dox. 5-Fluorouracil (5FU) is a well-established anticancer drug exerting cytotoxicity by affecting DNA synthesis and inhibiting cell proliferation [38]. In turn, 1,8-naphthalimides are fluorescent DNA intercalators binding preferentially to the A-D base pairs [39–41].

In this work, extending our recent studies on in vitro magnetic targeting of breast cancer cells (T47D) with purpurin- and 5FU-based Fe@MWCNT hybrids [42], we present anticancer activity and influence to selected cancer cells’ life cycle of various covalent and noncovalent “drug-nanotube” hybrids based on Purp, 5FU, and 1,8-naphthalimide DNA intercalators (NIDIs).

2. Results and Discussion

2.1. Synthesis and Physicochemical Properties of Nanotube Vehicles. Fe@MWCNTs were synthesized via a known c-CVD protocol using the highest possible concentration (9.6 wt.%.) of ferrocene in toluene as a feedstock [43] and were optionally oxidized with a nitrating mixture [17]. Morphology of the magnetically steerable nanotube vehicles was studied using SEM, TEM, and Raman spectroscopy (Figure 1). SEM image showed that Fe@MWCNT grew as vertically aligned nanotube films (also called “carpets” or
“forests”) (Figure 1(a)). The as-grown nanotube bundles display a significant free volume (in terms of the presence of interstices between the outer nanotube shells) reaching up to 90 vol.% and are practically indispersible in any solvent [44]. Individual Fe@MWCNTs were nonuniformly filled with iron nanoparticles visible in TEM image as from partially to continuously filled nanotube channels (Figure 1(b)). An inset in Figure 1(b) shows an extra-long, 0.5 μm long filling inside the nanotube channel. In a few cases, some onion-like graphitic excrescences containing iron nanospheres (removable under oxidation) could be found on the nanotubes’ outermost layers. The outer and inner Fe@MWCNT diameters (ODs and IDs) were nonuniform and varied not only between different but also for particular nanotubes; ODs and IDs were found to be equal to 44 ± 25 and 12 ± 6 nm, respectively. The length of pristine nanotubes was 100 ± 20 μm, while oxidation led to their partial cutting and the length was reduced to 50 ± 30 μm yielding nanotubes which fall into a biocompatible and water-dispersible category [19–21]. Raman spectrum of Fe@MWCNT (Figure 1(c)) shows three dominating signals at 1332, 1579, and 2662 cm⁻¹ which can be assigned to D-(disorder), G- (tangential graphite), and G′- (second-order
After oxidation of pristine Fe@MWCNTs for 20 min under reflux in a mixture of sulfuric acid (98%) and nitric acid (68%) (3/1, v/v) (for a detailed experimental protocol, please see Supporting Information (SI) available online at https://doi.org/10.1155/2017/1262309), the nanotubes changed their morphology into more twisted and entangled shape as visible by SEM (Figure 1(d)). This change took place due to extensive corrugation and formation of numerous grooves as revealed by TEM (Figure 1(e)). TEM images revealed that Fe@O-MWCNTs also contained encapsulated iron nanoparticles but, as determined by elemental analysis, the final Fe content dropped from 7.0 wt.% for Fe@MWCNTs to 3.0 wt.% whereas total O content (mainly as carboxylic groups) increased from 0 to ca. 18 wt.% [46]. The decrease in the Fe content after oxidative functionalization was observed due to partial erosion of the Fe nanoparticles from the nanotube tips and excrescences and, to a lesser extent, from the walls. Raman spectroscopy confirmed moderately destructive functionalization of graphene walls as the I_D/I_G ratio increased to 0.74 (Figures 1(c) and 1(f)).

Magnetic characterization of Fe@MWCNTs and their oxidized counterparts has revealed that their ferromagnetic behavior was a derivative of the multiphase structure of iron nanoparticles encapsulated in the nanotube cores. ⁵⁷Fe Mössbauer spectra with the superimposed fitted curves are shown in Figure 2 while the essential results are collected in Table 1.

The sample of Fe@MWCNTs was composed of three iron containing phases, that is, γ-Fe, α-Fe, and Fe₃C. The majority of iron (40 at.%) resided in the γ-Fe being paramagnetic at room temperature, 34 at.% of iron made ferromagnetic α-Fe, and the remainder (26 at.%) resided in the magnetically ordered iron carbide (Fe₃C). Spectra at high velocity range (±12 mm/s) were additionally acquired revealing absence of iron oxides. The sample of Fe@O-MWCNTs (of total Fe content equal to 3 wt.%) similarly contained metallic Fe phases as the dominating components but the content of Fe₃C (residing typically at the Fe/C interphase and hence being the most accessible and susceptible to aggressive oxidizing agents [49]) dropped to as low level as 5 wt.%. Nevertheless, it was clear from magnetic hyperfine field experiments that the concentration of ferromagnetic phases, that is, Fe and Fe₃C, exhibited maneuverability in the external magnetic field.

2.2. Drugs and “Drug-Nanotube” Hybrids. Purp, 5FU, and NIDIs were used as model anticancer drugs towards immobilization onto Fe@MWCNTs and Fe@O-MWCNTs (Figure 3) (detailed experimental procedures are presented in SI). Purp-N₁ (2a) was synthesized from Purp via its regioselective O'-alkylation with 1-azido-4-bromobutane in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a deprotonating agent. 5FU, after addition to 2-hydroxyethyl acrylate [50], was equipped with a longer linker in a reaction of the corresponding Michael adduct with 6-azidohexanoic acid, in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinum chloride (DMT-MM) as a condensing agent, yielding 5FU-N₁ (2b). Substituted 1,8-naphthalimide.
derivatives (2c-2f) were synthesized from the appropriate 1,8-naphthalic anhydrides via aminolysis with Boc-protected hexylenediamine (Scheme S1, SI). Purp-N$_3$ (2a) and 5FU-N$_3$ (2b) were covalently anchored to MWCNTs using nitrene chemistry yielding MWCNT$>$N-Purp (3aa) and MWCNT$>$N-5FU (3ab), respectively (Figure 3).

Pristine Fe@MWCNTs were oxidized to Fe@O-MWCNTs (bearing carboxylic groups) according to the protocol depicted earlier (20 min reflux H$_2$SO$_4$ (98%) + HNO$_3$ (68%) (3/1, v/v)) and the latter were subsequently conjugated with variously substituted 1,8-naphthalimide derivatives (2c-2f) using DMT-MM as a condensing agent and giving the appropriate hybrids (3bc-c-bf). Alternatively, Phth(CO)$_2$N-NH$_2$ (2c) treated with O-MWCNTs in DMF formed the "nanotubate" salt (3bc-i). The hybrids were obtained with various drug loadings (DLs) (Table 2) as determined by thermogravimetric analysis (TGA) (Figure 4).

DLs were calculated using a range of decomposition determined by projection of the peak onset and offset points (ca. 200 and 800°C) corresponding to minimal and maximal rates of decomposition of the "drug + linker" moieties in the "drug-nanotube" hybrids and further subtraction of the weight loss derived from the linker itself and volatiles (water and organic solvents used for the synthesis and isolation) [51–53]. From those values (for the same temperature ranges), weight losses (%) of MWCNTs and O-MWCNTs were subtracted yielding DLs for the particular "drug-nanotube" hybrids.

Anchoring the chemotherapeutic drugs (or their prodrugs) has left the morphology of the modified nanotubes intact. Nevertheless, this modification changes the surface polarity of nanotubes, their affinity to aqueous media, and hence further binding to cell membranes [54]. In order to visualize uptake and drug unloading from the "drug-nanotube" hybrids, fluorescent and scanning confocal microscopy studies were performed (Figure S1, SI). The observations were possible because all of the hybrids exhibited fluorescence in the spectral range of 4',6-diamidino-2-phenylindole (DAPI). It was found that "drug-nanotube" hybrids landed, pierced, or in a few cases penetrated cancer cells, similar to our previous studies [42], and the fluorescent drugs were unloaded intracellularly, mainly by diffusion after enzymatic cleavage or physical unloading. The enhanced cell penetration by nanotubes, also in the presence of magnetic field and nontoxic to human monocyte macrophage cells, was described by Boncel et al. [42] and Mahmood et al. [55]. The latter complex mechanism opens an additional route of intracellular release in the presence of a magnetic field. It was indeed confirmed that nanotubes, apart from altering cellular metabolic activity, may physically impair the membrane integrity yielding synergistic cytotoxicity [17].
With the aim of evaluating the cytotoxicity of nanotube vehicles, pure anticancer agents (or their prodrugs) as well as "drug-nanotube" hybrids, in the background of pure 5FU as a control drug, were analyzed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 5).

Both types of nanotube vehicles emerged as practically noncytotoxic against Me45 up to 50 µg mL⁻¹, whereas pristine MWCNTs (1a) were more cytotoxic for HCT116+ cells than O-MWCNTs (1b). For this cell line, inversely proportional concentration-viability dependence was observed. Moreover, HCT116+ cells were the most sensitive to nanotubes among all of the tested lines. Response of the Caco-2 line to the nanotubes falls in between the responses of Me45 and HCT+116. Me45 cells exhibited high in vitro resistance to "drug-nanotube" hybrids, similar to the nanotube vehicles. Only MWCNT>N-5FU hybrid (3ab) emerged as highly active against Me45 cells killing ca. half of their population at 50 µg mL⁻¹. Similar cytotoxicity was achieved by 5FU at 2000 times lower concentration. MWCNT>N-Purp (3aa) was the most cytotoxic against HCT116+ with ca. 60% of viable cells at the highest tested concentration. Caco-2 cells exposed to the "drug-nanotube" hybrids appeared to be the most suitable target for 1,8-naphthalimide-based intercalators anchored to nanotubes, with the 4-nitro derivative (3bf) as the most active one. Viability of the Caco-2 cell line at 50 µg mL⁻¹ of the MWCNT-CONH-N-(OC)₂Phth-4NO₂ hybrid (3bf) was below 35%. It must be emphasized that this effect was not achievable even at a high concentration of pure drug (2c) as 100 µg mL⁻¹. It seems that MWCNTs could exhibit intrinsic cytotoxicity by cellular membrane damage and, for example, interfere with the microtubule dynamics [56–58].

Furthermore, cytotoxicity studies of some of the tested drugs displayed a bimodal activity, particularly 2a, 2c, and 5FU (2g) against HCT116+ (Figure 5), which in general means higher toxicity at lower dose ranges and lower toxicity at higher doses. Such results are rather commonly known for pharmaceutical tests of different chemical compounds well known from the literature. For example, physiological

**Table 2: Drug loadings (DLs) in the "drug-nanotube" hybrids.**

<table>
<thead>
<tr>
<th>Nanotube</th>
<th>Drugs (and prodrugs)</th>
<th>&quot;Drug-nanotube&quot; hybrid</th>
<th>Drug + linker loading (wt.%)</th>
<th>DL (wt.%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCNT (1a)</td>
<td>Purp-N₃(2a)</td>
<td>MWCNT&gt;N-Purp (3aa)</td>
<td>15.0</td>
<td>11.8</td>
</tr>
<tr>
<td>O-MWCNT (1b)</td>
<td>M-Phth(NO₂)₂-N-NH₂(2d)</td>
<td>MWCNT-CONH-N-(OC)₂Phth-M (3bd)</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>3NO₂-Phth(NO₂)₂-N-NH₂(2e)</td>
<td>MWCNT-CONH-N-(OC)₂Phth-3NO₂ (3bc)</td>
<td>3.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4NO₂-Phth(NO₂)₂-N-NH₂(2f)</td>
<td>MWCNT-CONH-N-(OC)₂Phth-4NO₂ (3bf)</td>
<td>7.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* DL was calculated based on pyrolytic (Ar) TGA and it was calculated per pure drug (i.e., excluding linker) based on the structures in Figure 3.

**Figure 4:** TGA curves of nanotube vehicles and "drug-nanotube" hybrids; the analyses were performed under argon at 20 °C min⁻¹.


**2.3. Cytotoxicity of Drugs/"Drug-Nanotube" Hybrids, Induction of Apoptosis, and Influence on the Cancer Cell Cycle.**

With the aim of evaluating the cytotoxicity of nanotube vehicles, pure anticancer agents (or their prodrugs) as well as "drug-nanotube" hybrids, in the background of pure 5FU as
Figure 5: Cytotoxicity studies of nanotube vehicles (pristine Fe@MWCNTs and Fe@O-MWCNTs), anticancer agents, and "drug-nanotube" hybrids.
data obtained for hormone-like inhibitors tested in vivo on heartbeat frequency in insects suggested adaptation of cellular receptors at low concentrations of peptide leukemia-suppressor (for low heartbeat frequency, the inhibition was observed) and further their resistance at higher concentrations (at higher heartbeat frequency, no inhibition was observed) [59]. A similar phenomenon called hormesis is frequently found in the radiosensitivity or radioreistance assays where low doses of ionizing radiation are more toxic than higher ones, because of cancer cells adaptation to the toxic conditions at higher doses of IR [60]. Also, for star-shaped copolymers as nanocarriers of bioactive compounds tested on MCF-7 and MCF-7 doxorubicin-resistant cell lines, bimodal activities were observed: cytotoxicity decreased with increasing concentrations [61].

Apoptosis induced by nanotube vehicles and (preselected in the MTS assay) most cytotoxic hybrids was analyzed using an Annexin V assay (Figure 6). The results were presented in the background of pure anticancer agents (and their prodrug counterparts).

It is evident (Figure 6(a)) that the MWCNT>N-5FU (3ab) hybrid is characterized by the lowest tendency to induce necrosis in Me45. This behavior is in contrast to pure 5FU (2g) and 5FU-N1 (2b). Similar tendency could be found for the MWCNT>N-Purp (3aa) of which cytotoxicity was based on the activation of apoptosis (Figure 6(b)). Oppositely, 3bf hybrid induced necrosis in the highest fraction of Caco-2 population (Figure 6(c)). Obviously, in this context, MWCNT>N-5FU (3ab) and MWCNT>N-Purp (3aa) emerge as the most promising hybrids against Me45 and HCT116+ cells, respectively.

Analysis of the cell life cycles in the presence of nanotubes, drugs, and “drug-nanotube” hybrids was based on the determination of the cellular DNA content using propidium iodide (PI) and flow cytometry as a technique (Figure 7). The aim of the analysis, as a natural continuation of the above apoptosis studies, was to determine which phase of the cell division is impaired or inhibited by the most promising “drug-nanotube” hybrids.

As PI binds stoichiometrically to DNA; the number of DNA-PI complexes enables calculating histograms of DNA contents as a function of fluorescence intensity of the dye for nanotube vehicles, pure drugs, and “drug-nanotube” hybrids. Four main phases of the cell division [62, 63] could be observed here: sub-G1, G0/G1, S, and G2/M. The G2/M phase determines mitosis (in which the cell contains a double amount of DNA as before division, i.e., in the G0/G1 phase). The cells, described by phase sub-G1 in which DNA content is lower than in the G0/G1 phase, correspond to apoptotic and necrotic cells. Divided cells are in the S fraction. The level of polyploids (cells containing more DNA than in the G2/M phase) is typically referred to as noise and indicates damage that occurred during mitosis [64]. As shown, the results are in agreement with the apoptosis and cytotoxicity studies. All of the hybrids generated mostly apoptotic cells as a response. On the other hand, the presence of apoptotic cells does not exclude the possibility of cellular membrane damage while this effect can be significantly enhanced in the presence of a magnetic field.

3. Conclusions

In the quest for novel targeted drug delivery systems, “drug-MWCNT” hybrids were synthesized and their biological activity was determined in vitro against selected cancer cell lines. Nanotubes themselves, as vehicles enhancing targeting and revealing its intrinsic cytotoxicity, may be considered as potential platforms in the magnetic drug delivery. The earlier observed magnetic maneuverability [42] was here confirmed by Mössbauer spectroscopy which indicated the presence of
ferromagnetic Fe-based phases encapsulated in the core of nanotubes, also at a sufficient level in oxidized nanotubes. Their application however must be carefully addressed as different cancer cells reveal different levels of sensitivity to the “drug-nanotube” hybrids. Fe@MWCNT hybrids of 5FU and Purp, formed via nitrene chemistry, appear as the most suitable rapid response magnetic drug delivery systems in the locoregional therapy against Me45 and HCT116+ cells, while hybrids of oxidized Fe@MWCNTs and 4-nitro- and 4-(N-morpholinyl)-1,8-naphthalimide DNA intercalator formed via amidation against Caco-2 cells. The studies open a route to in vivo research against the so-preselected cancer cells.

4. Experimental

SEM analysis was performed by means of SEM JEOL 7001TTL. High-resolution transmission electron microscopy micrographs were obtained with a JEOL ARM 200F HRTEM with an accelerating voltage of 200 kV. The Raman spectra were obtained using a Ramascope-1000 spectrometer. The excitation source was a He-Ne laser of a wavelength of 633 nm. Each spectrum was collected with six accumulations of 10 s. Mössbauer transmission measurements were performed at room temperature using RENON MsAa-3 spectrometer equipped with the LND Kr-filled proportional detector and He-Ne laser-based interferometer used to calibrate velocity scale [65]. A commercial ^57^Co(Rh) source kept at room temperature was applied for 14.41 keV resonant transition in ^57^Fe. The Mössbauer absorbers were prepared using 100 mg of Fe@MWCNTs or Fe-O-MWCNTs and the absorbers’ thicknesses amounted to 50 mg/cm^2_. Data were processed by means of the Mosgraf-2009 software within the transmission integral approximation.

4.1. Stock Solutions/Dispersions. Pure compounds were introduced into media as solutions in DMSO (99.9%) and their cytotoxicity was determined for eleven concentrations, that is, 1 ng/mL, 5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 500 ng/mL, 1 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL. In order to obtain the appropriate concentrations, 1 mg of each compound was dissolved in DMSO (1 mL), and the mother solution (1 mg/mL) was used as a base for the more diluted solutions. Each solution was filtered through a 22 μm filter.

Cytotoxicity of nanotube vehicles and drug-nanotube hybrids was determined for dispersions of four concentrations, that is, 5, 10, 20, and 50 μg/mL. Similarly, as for pure compounds, firstly, the mother dispersion of 1 mg/mL was prepared by 1 h of ultrasonication at 37°C, and then diluted and ultrasonicated dispersions were prepared and introduced into the media.

4.2. Cell Lines. Three cell lines were used in the studies, namely, Me45, HCT116+, and Caco-2. The first cell line is human fibroblasts derived from malignant melanoma. The two other epithelial lines derive from human colon cancer.

All of the cells are characterized by the adherent growth mode. Human melanoma (Me45) cell line was derived in 1997 from melanoma metastasis to lymph node of a 35-year-old male patient treated at the Institute of Radiobiology of the Maria Skłodowska-Curie Memorial Cancer Centre, Gliwice (Poland). Human colon carcinoma (HCT116+) cell line is a line of human colon adenoma cells. The cells were grown in the bank of Maria Skłodowska-Curie Memorial Cancer Centre, Gliwice (Poland).

Human Caucasian colon adenocarcinoma (Caco-2) cell line was isolated from a 72-year-old Caucasian male. The line was purchased from the European Collection of Cell Cultures (ECACC) and the line was generously given by the Medical University of Silesia in Zabrze (Poland). The cells are of high resemblance to the human intestinal epithelium cells. It must be emphasized that Caco-2, as the only one from all of the cell lines previously derived from the intestinal tumor line, is capable of forming a layer of cells possessing morphological, structural, and functional similarity to the intestinal epithelium occurring in the human body.
(a) formation of intercellular tight junctions, (b) expression of specific enzymes, for example, alkaline phosphatase or protein transport channels (P-glycoprotein, cytochromes, etc.), and (c) cell differentiation towards the formation of microvilli of the "brush border."

4.3. Cell Cultures. All cell lines were cultured in culture flasks with 25 cm² (T-25) or 75 cm² (T-75) bottom surface. Basic medium DMEM/Ham's F-12 with L-glutamine was supplemented with Fetal Bovine Serum (FBS) (12%) containing antibiotics MycoKill AB (1:50 dilution). The culture was grown in an incubator under standard conditions, that is, 37°C, 5% CO₂ content in the atmosphere of 5%, and humidity of ca. 60%.

4.4. Passage and Culture of Cells. Under sterile conditions, the stale medium was poured from the above cell cultures. The bottom of the bottle was washed with 1 or 3 mL of a concentrated solution of trypsin, for T-25 or T-75 bottles, respectively, in order to neutralize the trypsin present in the culture medium and to remove dead cells only slightly adhering to the bottom of the bottles. Then, 3 mL (T-25) or 5 mL (T-75) of a freshly prepared concentrated solution of trypsin was added to the bottles using a serological pipette and the bottles were placed in the incubator for a few minutes. The process of separation of cells was continuously monitored under a light microscope. The duration of the process varied and depended on the adhesion degree of cells to the substrate and morphology of the cells (in the case of HCT116+ and Caco-2 cells, the process was rapid, whereas Me45 cells were detaching very slowly). After complete detachment of the cells, an equal amount (3 or 5 mL) of the fresh culture medium was added to the bottle. The bottle's content was carefully divided using a serological pipette, transferred to Falcon tubes, and then centrifuged in a bench centrifuge (1500 rpm, 3 min). The medium with trypsin was carefully decanted from the cell sediment, and then 1 mL of fresh medium was added again. After precise distribution of the cell sediments in the new medium, ca. 100 μL of suspension was collected in an Eppendorf tube in order to count the number of cells in the Bürker counting chamber. After the passage, the bottle was refilled with fresh culture medium DMEM (8-9 mL T-25) and (10–12 mL T-75) and the growth of cells was continued.

After determination of concentration of cells per 1 mL of suspension, the appropriate volume of suspension was withdrawn to a new test tube and filled up with fresh DMEM medium, depending on the volume required for the planned assay.

4.5. Cytotoxicity Assays. The cells were trypsinized, counted in the Bürker chamber, suspended in a suitable amount of DMEM medium, and then placed in 96-well plates, which were then cultured for 24 h under standard conditions (37°C, 5% CO₂). After 24 h, the medium was removed and replaced with a fresh one of volume allowing for dosing solutions/dispersions of tested compounds, nanotube vehicles, “drug-nanotube” hybrids, and the control of the appropriate concentration. Each measurement was performed in triplicate.

After the addition of media and solutions/dispersions, the plates were incubated for 24 h under standard conditions. Then, the medium was removed from above the cells and was replaced by 100 μL of phosphate-buffered saline (PBS). The MTS reagent was combined with 1-methoxyphenazine methosulfate (PMS) (2 mL MTS + 100 μL PMS for each plate) and 20 μL of the mixture was added to each well. The plates were incubated for 2 h until the color in the control wells was changed from light yellow to brown. The absorbance for formazan produced solely by alive cells was measured at a wavelength of λ = 490 nm using a microplate spectrophotometer. The absorbance values were expressed as a percentage change in viability of tested cells relative to the control cells. Cell viability was therefore calculated from the formula cell viability = A/A₀ · 100%.

4.6. Analysis of Apoptosis. After removal of cells, trypsin was neutralized with the previously collected medium. The samples were centrifuged (1500 rpm, 3 min) and the supernatant was carefully removed using a pipette. The precipitate was washed with 300 μL of PBS and, to the rinsed precipitate of cells, cold Annexin-dedicated buffer (50 μL) and then Annexin V (2.5 μL) were added. Next, the open tubes were placed into the incubator for 20 min. After this time, 250 μL of Annexin-dedicated buffer and 10 μL of PI solution (3 mg/mL) were added and the whole system was mixed carefully. The samples were incubated in the dark for 15 min and then transferred onto ice. Immediately prior to the flow cytometric analysis, the samples were vortexed and transferred to cytometer tubes. The counting camera encountered first 10,000 events. The results were analyzed using Flowing MS Office® ver. 2.5.0 and MS Excel 2007®.

4.7. Cell Cycle Analysis by Flow Cytometry. After addition of the appropriate amounts of medium and solutions/dispersions, the samples were incubated for 24 h under standard conditions. Afterwards, the medium was moved to new Eppendorf tubes, whereas the remaining cells were detrypsinized (by adding 500 μL of concentrated trypsin solution to each well). Trypsin was neutralized by previously poured off medium containing dead cells and the samples were centrifuged (1500 rpm, 3 min). The supernatant was carefully poured off, and the precipitate was rinsed with 300 μL of PBS. Then, to each tube, 600 μL of frozen 96% EtOH was added dropwise and the tubes were vortexed for the accurate fixation of the cells. The samples were stored at 4°C for a minimum of 24 h, and up to a week and before the measurements, they were again centrifuged (1000 rpm, 3 min). The supernatant was collected and the residual cells were rinsed with 300 μL of PBS. Then, 50 μL of RNase solution at a concentration of 100 μg/mL (prepared from a stock solution of 10 mg/mL, in a ratio of 1: 49 RNase/PBS) was added in order to remove RNA and the samples were shaken in a Thermoblock at 37°C for 20 min. Then, 250 μL of PI was added at a concentration of 100 μg/mL and the samples were allowed to stand in the dark for 10 min. After this time, the samples were transferred onto ice. Immediately prior to the measurements, each sample was vortexed thoroughly and the tube was placed in a flow cytometer. The counting camera
4.8. Statistical Analysis. All measurements for cytotoxicity, apoptosis, and cell cycle were performed at least in triplicate, and results were presented as average +/- SD. The significance of any changes, according to the control and untreated cells, was calculated with Student’s t-test with p value < 0.001. Relevant changes presented in Figures 5–7 were indicated by an asterisk (*).

4.9. Preparation of the Samples for Microscopy Imaging. The sample preparation for microscopic analysis lasted 72 h. Firstly, cell trypsinization was performed, the amount of cells sample preparation for microscopic analysis lasted 72 h. EtOH (10 min). After fixation, alcohol was removed, 500 μL PBS and then fixed with 70% EtOH (10 min). After fixation, alcohol was removed, 500 μL of deionized water was added, and the system was allowed to stand for 2-3 min. Subsequently, water was removed with a pipette and the well plate was broken off. To each well, 10 μL of DAPI was introduced and a drop of glycerol was added, and then the well was covered with a coverslip, which was then protected against drying using a dedicated resin. The samples were stored in closed boxes at 4°C.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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